DETERMINANTS OF ANTI-RETROVIRAL THERAPY DRUG-RELATED TOXICITIES IN HIV POSITIVE PATIENTS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine (MD)

By

Mohammed Ibn-Mas'ud, Danjuma

October 2017

Abstract

Antiretroviral therapy (ART) drugs have increasingly been shown to contribute significantly to the morbidity of HIV positive patients exposed to them. This is more so with increasing survival of HIV patients since the introduction of ART. Individual ART drugs have been associated with specific organ related toxicities and clinical toxicity syndromes, such as Tenofovir disoproxil fumarate (TDF) association with risk of kidney injury. The pattern, pathogenesis, as well as key genetic and non-genetic determinants of some these clinical ART drug-related toxicity syndromes remains uncertain.

This thesis sets out to investigate the pattern/clinical/laboratory phenotype of ART drug related kidney toxicity, including the role of emerging biomarkers that define and most accurately diagnose these toxicity syndromes. I, and my co-reviewer (Dr Sudeep Pushpakom, University of Liverpool) then systematically reviewed, and carried out a metanalysis of current evidence with regards to the reported genetic association between TDF exposure, and risk of kidney related toxicity. I further explored a population cohort (MHRA) to ascertain the pattern/clinical phenotype of kidney injury following exposure to TDF in these cohorts of patients, and determine any discernible variation as it relates to what has been reported from clinical trial cohorts. Additionally, I examined the association between single nucleotide polymorphisms (SNPs) of genes encoding protein transporters involved in the bio-disposition of TDF in HIV positive patients, with kidney injury following exposure to ART drugs.

Specifically, in an attempt to explore the pattern of kidney injury in HIV patients exposed to TDF in observational databases, I reviewed 407 yellow card records of HIV positive patients on TDF who developed kidney injury and had them reported to

MHRA. One hundred and six (106) of these satisfied criteria for TDF related kidney injury, of which 53 (50%) had features of kidney tubular dysfunction (KTD), 35 (33%) were found to have features of glomerular dysfunction, and 18 (17%) had Fanconi syndrome. The median TDF exposure was 316 days (IQR 120-740). The incidence of hospitalisation for TDF kidney adverse effects was high, particularly amongst patients with features of Fanconi syndrome.

To define the clinical phenotype (including surrogate markers) of ART drug related kidney injury, I recruited and investigated the diagnostic utility of KIM-1/Cr (corrected for urinary creatinine excretion) in a 114 cross-sectional cohort of HIV positive patients (104 "on" ART, and 10 "off" ART drugs). HIV positive patients both "on" and "off" ART drugs had a higher baseline median (≥4.17ng/mg), upper quartile (≥8.6ng/mg), and urinary KIM-1/Cr levels compared to either non-HIV positive normal volunteers (0.39 ng/mg), or those with acute kidney injury in the general population (0.57 ng/mg). By ROC analysis, KIM-1/Cr (ng/mg) had a higher AUC (0.67) compared to either serum creatinine (0.64) or eGFR (0.31) in diagnosing patients with kidney injury. When median KIM-1/Cr (≥4.17ng/mg) was utilised as a marker of kidney injury, TDF exposure (per year increase) was significantly associated with risk of kidney injury in multivariate analyses (Odds ratio 1.4, CI 1.02-1.82, P = 0.034). In a candidate gene based-based approach, I subsequently investigated the strength of association between the *ABCC2* and *ABCC10* sub-family genetic polymorphisms, and risk of kidney injury in HIV positive patients exposed to TDF. Patients with KTD had higher current CD4 cell counts, lower eGFR, and were less likely to possess the genotype *CC* at position 24 of the *ABBC2* (MRP2, rs717620) gene. In multivariate analysis, genotype *CC* at position 24 of the *ABBC2*

gene (odds ratio =0.05, 95% confidence interval = 0.003-0.7, P = 0.027) was significantly associated with reduced risk of KTD.

These findings support the observation that ART dugs are a leading cause of organ specific morbidity including TDF related kidney injury. Furthermore, we have demonstrated higher thresholds of low molecular weight proteinuria (including RBPCR and KIM-1/Cr) following TDF exposure, in HIV positive patients with normal kidney function (normal eGFR).

Further work is required in prospective patient populations to validate some of the findings in our study including the potential diagnostic utility of low molecular weight proteinuria (KIM-1 and RBP) in these cohorts of patients. Additionally, there will be need to explore the clinical significance of possession of the *ABCC2 24CC* (rs717620) and *ABCC10* SNP's in an appropriately defined patient population

Declaration

I hereby declare that this dissertation represents my original work, that where other people's expressions or language were used, quotation marks are indicated and appropriate credit given. I also declare that this dissertation describes original work and has not been previously presented for the award of any other degree of any institution.

I developed the clinical/research question Chapter 2 (systematic review) of this thesis explored. This was carried out with both infrastructural and intellectual support from my supervisors. I was primarily involved in data extraction, evaluation, and further analyses, and was supported by my co-reviewer Dr Sudeep Pushpakom. Dr Ben Frances of the Department of Biostatistics, University of Liverpool provided further statistical input including assistance with appraisal of the evidence.

I conceived and developed the research question Chapter 3 (Examination of MHRA population database) of this thesis sought to address with guidance from my supervisor. I specifically carried out retrieval, classification, and analyses of data from MHRA with input from my primary supervisor.

Chapter 4 (Candidate gene-based association study on *ABCC2, ABCC4, ABCC10, OAT1,* and *OAT3* sub-family polymorphisms) was developed working with both my primary and secondary supervisors including inputs from Dr Frank Post at Kings College Hospital London, United Kingdom (UK). I carried out extraction of genomic DNA from serum samples with support of Ms Deirdre Egan (Department of Molecular and Clinical Pharmacology, University of Liverpool). Additionally, I carried out DNA quantification and genotyping (using *Taqman* assays) of *ABCC2, ABCC4, ABCC10, OAT1,* and *OAT3* single nucleotide polymorphisms (SNP). Further statistical analyses of clinical and SNP data was carried out by me with

support from Dr Sudeep Pushpakom (the Department of Molecular and Clinical Pharmacology, University of Liverpool).

Chapter 5 (DETIKI clinical cohort) was conceived by me and further developed with inputs from my supervisor (Professor Saye Khoo). Primarily, I carried out patient recruitment into this clinical cohort, with additional recruitment support from Dr Nurul-Huda Mohamad-Fadzillah. I prepared, centrifuged (where necessary), and classified study samples for storage. I carried out biomarker assays including KIM-1, and urine creatinine assays with guidance from Dr Steve McWilliams (Wolfson Centre for Personalised medicine, the University of Liverpool). I carried out cleaning of study data including abstraction of patient electronic records. Further, data analyses were jointly carried out by myself, and my supervisors (Professor Saye Khoo and Dr Dan Antoine).

However, the overall concept, textual presentation, various analyses, and inferences drawn or expounded in this thesis, wholly represent my own views and interpretation. Both the clinical and laboratory sections of this research were carried out in the Department of Molecular and Clinical Pharmacology at the University of Liverpool, and Royal Liverpool and Broadgreen University Hospital, Liverpool, the United Kingdom.

Mohammed I Danjuma

Acknowledgements

I will like to thank my supervisors Professor Saye Khoo, Professor (Sir) Munir Pirmohamed, and Dr Dan Antoine for their guidance, as well as time, and intellectual resources they have invested in both my clinical training, and development of this thesis.

I also owe a profound debt of gratitude to the Royal Liverpool and Broadgreen University Hospital for providing the enabling environment for patient recruitment into various studies that constitute this thesis. Additionally, I will like to thank patients in these hospitals for consenting to participate in these studies and have their samples utilised for this invaluable research work.

I am grateful to the clinical authorities and patients at the Kings College Hospital London for allowing the use of their samples for this work. The contribution of Dr Frank Post is particularly notable in this regard, especially in the light of his time investment, as well as intellectual guidance in the Biomarker section of this research.

Furthermore, I will like to acknowledge the contribution of laboratory and academic staff at both the Pharmacology research laboratories at H block, BAF, as well as Wolfson Centre for Personalised Medicine here at the University of Liverpool for their technical and academic support in the development of various assays that constitute this research work. Notable amongst these includes but not limited to Deirdre Egan, Helen Reynolds, Dr Steve McWilliams, Dr Neil Liptrott, Professor Andrew Owen, and Professor David Back amongst many others whose contribution have been tremendously useful to the success of this work.

Finally I will like to thank my Family for their unquestionable support most notably those of my Mother (Hajara), and my brother Alhaji Hamza Elayo Mohammed for

always been the source of pride, support, and above all guidance in all my life journeys thus far.

Table of Contents

List of Tables

List of Figures

Publications

Danjuma M I et al. An investigation of the pattern of kidney injury in HIV-positive persons exposed to Tenofovir: An examination of the MHRA database. *Int J STD AIDS* 2014; 25(4): 273-9

Danjuma M I, Mohiuddin A, Pirmohamed M, and Khoo S. Tenofovir (TDF) and the Kidney in HIV-infected patients: The evidence thus far. *HIV Therapy* 2010; 4.3: 345-359

Danjuma MI: Pharmacokinetics of non-nucleoside reverse transcriptase inhibitors. *HIV therapy* 2009; 3: 625-632

Abbreviations

A		**CNS**	Central nervous system
ABC	Abacavir	**COBI**	Cobicistat
ABCC2	ATP binding cassette sub family C, member 2	**CONSORT**	Consolidated standards of reporting trials
ABCC4	ATP binding cassette sub-family C, member 4	***CYP***	Cytochrome
ABCC10	ATP binding cassette sub-family C, member 10	**CCR5**	C-C chemokine receptor type 5
ACR	Albumin creatinine ratio	***CFTR***	Cystic fibrosis transmembrane conductance regulator
ACTG	AIDS clinical trials group	**CRF**	Case record form
ADR	Adverse drug reaction	**CVD**	Cardiovascular disease
AE	Adverse event	**CVS**	Cardiovascular system
AKI	Acute kidney injury	**D**	
AIDS	Acquired immune deficiency syndrome	**DETIKI**	Determinants of kidney disease
ART	Antiretroviral therapy	**DRV**	Darunavir
AUC	Area under the curve	**DD1**	Didanosine
ATV	Atazanavir	**DDC**	Zalcitabine
B			
BHIVA	British HIV association	***DNA***	Deoxyribonucleic acid
C		**DRESS**	Drug reaction with eosinophilia and systemic symptoms
cART	Combination antiretroviral therapy	**E**	
Cmin	Minimum concentration	**EFV**	Efavirenz
CD	Cluster of differentiation 4	**ETR**	Etravirine
CXCR4	C-X-C chemokine receptor type 4	**EVG**	Elvitegravir
CYP3A4	Cytochrome 3A4	**F**	
FDA	Food and drug administration	**LPV**	Lopinavir
FTC	Emtricitabine	**M**	

G	
Gp120	Glycoprotein 120
GWAS	Genome wide association study
H	
HapMap	Haplotype map
HDL	High density lipoprotein
HIV	Human immune deficiency virus
HLA	Human leucocyte antigen
HRS	Abacavir hypersensitivity syndrome
HWE	Hardy Weinberg equilibrium
I	
IDV	Indinavir
IL-18	Interleukin-18
InSTI	Integrase strand transfer inhibitors
K	
KTC	Kidney tubular cells
KTD	Kidney tubular dysfunction
KIM-1	Kidney injury molecule-1
L	
L-FABP	L-type fatty acid binding protein
LA	Lipoatrophy
LH	Lipohypertrophy
LDL	Low density lipoprotein
LMWP	Low molecular weight proteinuria
R	
RBP	Retinol binding protein
RNA	Ribonucleic acid
ROC	Receiver operator curve
RTV	Ritonavir
SLC	Solute carrier gene
SREBP-1	Sterol regulatory element binding protein-1
MAF	Minor allele frequency
MDR	Multi-drug resistant
MtDNA	Mitochondrial DNA
mRNA	Messenger RNA
MRC	Medical research council
MRP2	Multi-drug resistant protein-2
MRP4	Multi-drug resistant protein-4
MRP7	Multi-drug resistant protein-7
MVC	Maraviroc
N	
NAG	N-acetyl-β-D glucosaminidase
NGAL	Neutrophil gelatinase associated lipocalin
NRTI	Nucleoside reverse transcriptase inhibitors
NNRTI	Non-nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
O	
OAT	Organic anion transporter
P	
PK	Pharmacokinetics
PD	Pharmacodynamics
PIC	Pre-integration complex
PI	Protease inhibitor
PCR	Polymerase chain reaction
PCT	Proximal tubular cell
TAF	Tenofovir alafenamide
TK2	Thymidine kinase 2
TDF	Tenofovir disoproxil fumarate
TFV	Tenofovir (active moiety)
TmPO4	Tubular maximum for phosphate reabsorption
U	
UGT1A1	Uridine diphosphate glucoronyl transferase
V	

SNP	Single nucleotide polymorphism	**VL**	Viral load
SQV	Saquinavir	**W**	
TAF	Tenofovir alafenamide fumarate	**WHO**	World Health Organization

CHAPTER ONE

GENERAL INTRODUCTION

1.0 Introduction to HIV virus and antiretroviral drugs

This is a review of the biology of HIV virus, and the spectrum of ART drugs currently in use as part of various treatment regimens.

1.1 Historical context

The clinical syndrome of acquired immune deficiency syndrome (AIDS) is caused by a retrovirus called human immune deficiency virus (HIV) (1). Since the initial characterisation of this disease in 1981 and subsequent discovery of the virus responsible for it in 1983, there have been unrelenting efforts at understanding of both the biology of the disease/virus and pharmaco-therapeutic targets to exploit for treatment. Despite initial conflicting reports as to the likely origin of the virus, the most accepted explanation is its transmission from chimpanzees to humans (2). Chimpanzees carry a virus (simian immunodeficiency virus) that bears some biological similarities with HIV and this was probably transmitted to humans following contact between hunters and infected simian blood (2).

1.1.1 Global epidemiology of HIV/AIDS

The World Health Organisation (WHO) 2013 Global Health Observatory data reported a case burden of about 78 million people have been infected with the HIV virus since the beginning of the epidemic, with a case mortality of about 39 million people thus far (3). It presents a global adult (15-49 years) prevalence of about 0.8 % (0.7-0.8%), with the highest prevalence rate in sub-Saharan Africa (4.5%). This accounts for about 71% of the case burden worldwide (3). Although the number of AIDS cases in the United Kingdom (UK) has fallen considerably since its peak between 1993-1995, the 2013 "HIV in the United Kingdom report" (4) puts the case burden of people living with the disease at 98,400 (93,500-104,300), figure 1.1. This gives an overall prevalence rate of about 1.5 per 1000 population (1.0 in women, and

2.1 in men). Of these, one in five (21,900, 22% [18%- 27%]) were undiagnosed and unaware of their infection (5). Figure 1.1

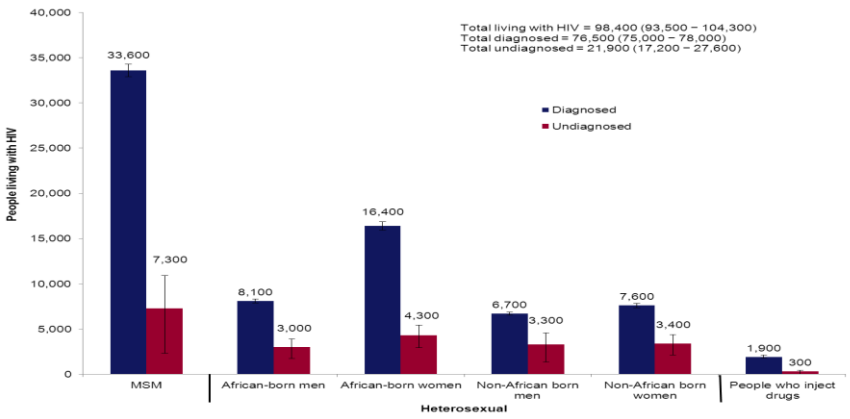


Figure 1.1: People living with HIV (both diagnosed and undiagnosed): UK, 2012 [Adapted from HIV and AIDS Reporting Section, Centre for Infectious Disease Surveillance and Control] (5)

1.1.2 HIV Transmission

HIV is transmitted through a number of routes including sexual intercourse, infected needles, blood transfusion, vertically from infected pregnant mothers, and during delivery or breastfeeding (6). There is marked geographical variability in these various modes of transmission. In Europe/UK for example, migration of infected patients from sub-Saharan Africa contributes substantially to the case burden there, accounting for about 36% of all new cases in 2006 (5). In sub-Saharan Africa however, heterosexual transmission accounts for a significant number of new cases (7). Regardless of the mode of transmission, without treatment (in some individuals) the clinical syndrome of AIDS develops in about 10 years from index infection (7), with death supervening following opportunistic infections. It is noteworthy that despite a rise in number of new cases, the mortality due to HIV/AIDS has recently continued to witness a sustained decline (figure 1.2)

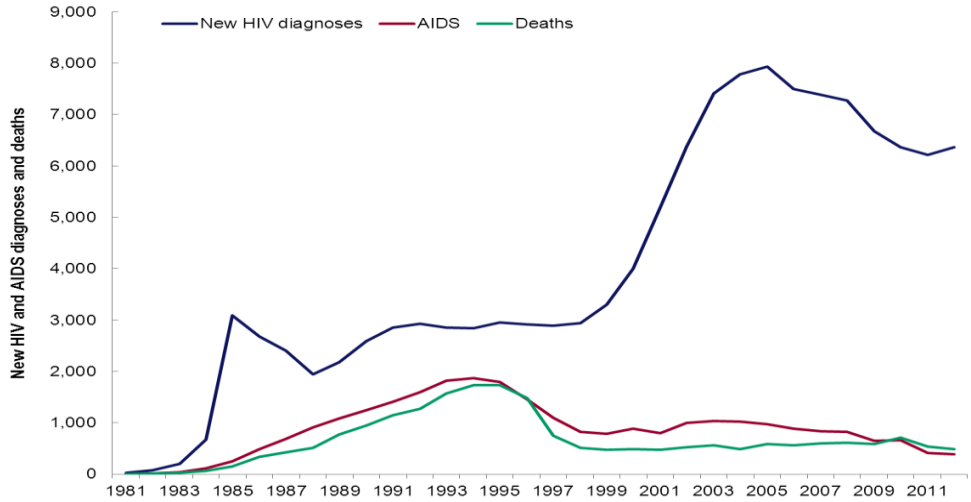


Figure 1.2: Annual new HIV/AIDS diagnoses and deaths: UK, 1981-2012 [Adapted from HIV and AIDS Reporting Section, Centre for Infectious Disease Surveillance and Control] (5)

1.1.3 Pathophysiological mechanisms

HIV is a lentivirus (retroviridae family) that exhibits tropism for immune-competent cells especially helper CD4+ T-lymphocytes, macrophages, and microglial cells (in the central nervous system) (8). Of the two characterised variants (HIV types 1 and 2), HIV-1 cause most of the infections in Europe and has further sub-divisions into sub-types called clades (8). The latter is associated with significant and important geographical variation (9). HIV infection begins by initial entry of the virus into CD4+cells and other immune competent cells mediated by interaction of its glycoprotein envelope receptors (gp120) with receptors on surface of these cells (10). However, other variants including M-tronic strains of HIV-1 and non-syncytial inducing strains (R5 viruses) initiate infection by additionally utilising a membrane-spanning β-chemokine co-receptor (CCR5) for this initial entry (11). Syncytial inducing viruses (X4 viruses) on the other hand utilise the α-chemokine co-receptor (CXCR4) for entry into both CD4+ T-cells and macrophages (11). Following fusion of the viral envelope proteins with receptors on immune competent cell, HIV

genomic RNA is released into the cell following uncoating of the viral core (12). Reverse transcription involves use of viral RNA polymerase to catalyse synthesis of viral DNA copies (utilising deoxyribonucleoside triphosphates [dNTPs] substrates) from genomic viral RNA (13). Reverse transcription leads to formation of viral pre-integration complex (PIC) which is made of viral DNA copies as well as other host proteins (13). This then migrates to the nucleus where viral *Integrase* enzyme catalyses the integration of viral DNA into host DNA (14). Viral mRNAs are subsequently transcribed, and translocated to the protein assembly complex in the host cell cytosol. HIV viral structural polyproteins thus generated are cleaved by protease enzyme in the course of the maturation process. This result in formation and budding off of infective mature HIV virions to initiate new cycle of infection of other immune-competent cells (15). Figure 1.3 shows schematic representation of the HIV replication process.

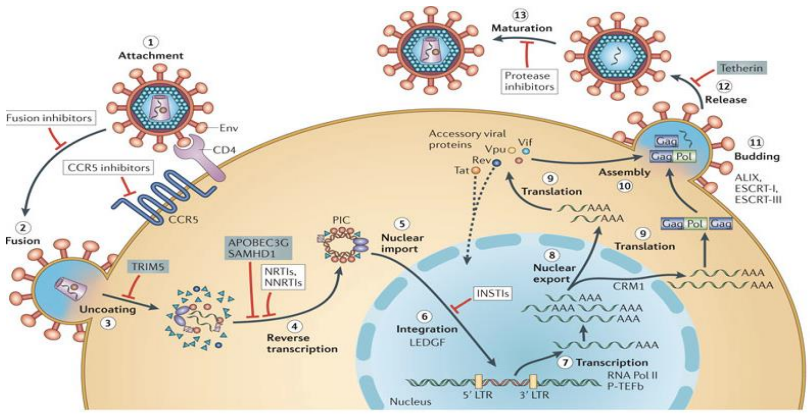


Figure 1.3: Schematic representation of pathogenetic mechanisms involved in HIV infection [adapted from Engelman A *et al* (16)].

1.2 Antiretroviral drug classes

The various stages of HIV replication cycle present excellent therapeutic targets that have allowed for development of drugs that have evidently increased the life

expectancy of people infected with HIV (17). These groups of drugs are called antiretroviral therapy drugs, and are made of a number of drug classes used in various combinations/regimens (of 2 to 3 drugs). And as suggested by systematic studies, they have been adapted by national and international treatment guidelines (18). The choice of a particular drug class or regimen is dependent on a number of factors including ease of administration, adverse effects profile, genotype testing profile, associated co-morbidities, as well as food/drug-drug interactions amongst others (18). The following is a review of the pharmacokinetic, pharmacodynamics, and peculiar chemical characteristics of these drugs that may play a role or help to explain some of their reported adverse reactions.

1.2.1 Nucleoside reverse transcriptase inhibitors (NRTI's)

These represent the first class of drugs approved for treatment of HIV/AIDS and continue to form an integral part of most national and internationally recommended treatment regimen (18). They include Zidovudine (AZT), Didanosine (DDI), Zalcitabine (DDC), Stavudine (d4T), Lamivudine (3TC), Emtricitabine (FTC) Abacavir (ABC), Adefovir, and Tenofovir disoproxil fumarate (TDF) (Figure 1.4). They are administered as pro-drugs but undergo cellular kinase mediated phosphorylation upon host cell entry to exert their anti-viral effect on HIV RNA viral synthesis (19). NRTI's have no 3'hydroxy group at their 2' deoxyribosyl sugar moiety (20). The implication of this is that the critical 3'5' phosphodiester bond between them and incoming 5' nucleosides triphosphates cannot be formed leading to chain termination. Both RNA-dependent DNA, and DNA-dependent DNA synthesis are affected resulting in inhibition of synthesis of both (positive and negative) strands of HIV proviral DNA (20, 21). They are competitive substrate inhibitors of HIV reverse transcriptase (22). Additionally, they also inhibit

mitochondrial DNA (mtDNA) polymerase gamma (γ) resulting in depletion of mtDNA precursor pool and a number of adverse effects including accumulation of lactic and pyruvic acids (23).

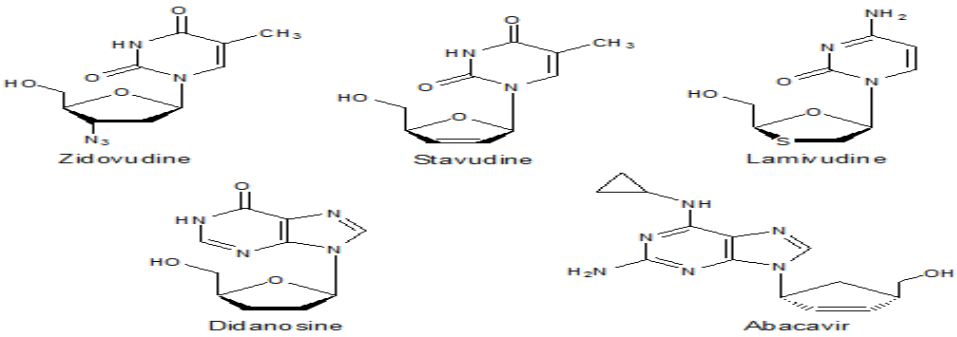


Figure 1.4: Molecular structure of some nucleoside reverse transcriptase inhibitors (NRTI's)

Long-term experience with these agents meant that our understanding of their adverse effects profile is relatively better and more comprehensive than newer agents. Whilst no class effects have been attributed to any of their adverse effects, the underlying pathophysiological mechanism in all of them appears to be varying degrees of mitochondropathy (22). In a mechanistic study, Hein et al (24) showed that down-regulation of mitochondrial thymidine kinase 2 (TK2), and deoxyguanosine kinase (dGK) by NRTIs such as DDI might represent the mechanism through which they mediate their toxicity (24). This was thought to result in depletion of mitochondrial DNA (mtDNA) precursor pools, depletion of mtDNA, mitochondropathy, and long-term organ/system specific adverse effects (22, 24). Amongst reported adverse effects associated with this class of drugs includes

pancreatitis, hepatitis, lactic acidosis, bone marrow suppression, myopathy Cardiomyopathy, and lipodystrophy (24).

Reports thus far have linked NRTI associated pancreatitis mostly to exposure to DDI and D4T (25, 26). The underlying pathophysiological mechanism is still uncertain but a role for carriage of the cystic fibrosis trans-membrane conductance regulator (CFTR) has been suggested (27, 28). Indeed a pharmacogenetic study exploring this role in HIV positive patients showed that up to 40% of patients with hyperamylasemia and clinical pancreatitis were carriers of the CFTR gene (28). The overall incidence of clinically relevant pancreatitis related to NRTI's is variable. An evaluation of patient level data of 20 clinical trials by Reisler et al reported an overall incidence of about 0.85/100 persons/year (29). The rates of pancreatitis were highest amongst patients on Indinavir/DDI/D4T regimen (29).

1.2.2 Non-nucleoside reverse transcriptase inhibitors (NNRTI's)

Despite reported treatment-limiting adverse effects, these agents have formed an integral part of the backbone of most ART treatment regimen (18, 30) (Figure 1.5). They act primarily by prevention of HIV replication through non-competitive inhibition of the multifunctional HIV-1 reverse transcriptase (RT) enzyme (31). This subsequently affects the catalytic activity of the enzyme resulting in inhibition of viral replication (31). Other secondary modes of action have been suggested for NNRTI's affecting various stages in the reverse transcription process including their effect on RT RNase-H (32). *Rnase-H* is part of the multifunctional complex of the RT enzyme, other functional units includes RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities. Despite their disparate chemical identities, NNRTI's bind to the same site on reverse transcriptase enzyme, with each NNRTI interacting with different amino acid in the binding pocket (31). They show

diverse pharmacokinetic profiles resulting in differences in efficacy, differing levels of drug exposure (*AUC*), and marked inter-individual and intra-individual variability. They are generally well absorbed following oral administration (33, 34, 35).

Nevirapine (NVP) is a dipyridodiazepinone (11-cyclopropyl- 5, 11-dihydro-4-methyl-6H-dipyrido [3, 2-b: 2', o; 3'-e] [1, 4] diazepin- 6-one) compound. It is rapidly and almost completely absorbed within 4 hours following oral administration of a 200mg tablet (33). Despite the effect of food on its absorption, NVP achieves a bioavailability of 93%, 63% of this is bound to plasma proteins particularly albumin (33). Kidney impairment have no significant effect on plasma NVP concentration, therefore no dose adjustment is advised in patients with mild, moderate or severe kidney failure (33). However in patients with end stage kidney disease on dialysis, there is a significant decrease in *AUC* of NVP. Consequently administration of a supplementary dose of 200mg of NVP is advised after each dialysis session in these cohorts of patients (33). Whilst caution is advised with administration of NVP in patients with mild to moderate liver injury, its use in those with severe liver injury (child-Pugh class C) should be avoided (33).

Efavirenz (EFV) has excellent bioavailability and is extensively distributed in body fluids (34). It exhibits linear pharmacokinetics with a prolonged half-life of 40- 50 hours at steady state (34). This makes administration of single daily oral dose possible, resulting in attainment of steady state within 2 weeks of initiation of therapy. An insignificant concentration of EFV (>1%) is excreted unchanged in urine. EFV causes mild transaminitis (2%), with caution advised when used as part of ART regimen in patients with mild to moderate liver injury (34). Its use is however contraindicated in patients with severe liver injury (child-Pugh class C) (34).

Etravirine (ETR) is a second generation NNRTI (diarylpyrimidine) which inhibits viral replication through binding directly to HIV reverse transcriptase disrupting the active site of the enzyme (35). Food significantly affects its absorption reducing its *AUC* by about 50%, therefore the drug is advised to be taken after meals (35). Owing to limited excretion in urine, dose adjustment is not advised in patients with kidney failure (35). Mild to moderate hepatic impairment results in about 18% reduction in some PK parameters including *AUC* and *Cmin* with no effect on its dosing regimen (35). However, its use in patients with severe liver impairment is contraindicated because of limited data (35).

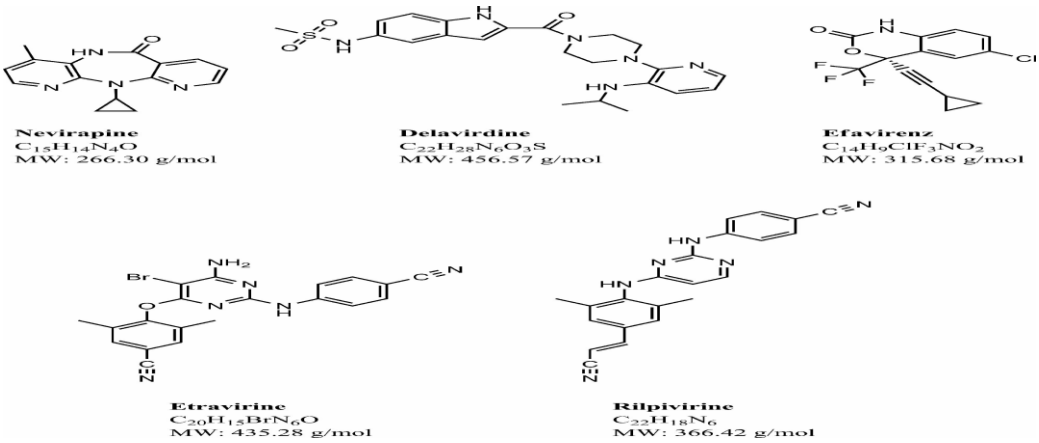


Figure 1.5: Molecular structure of some clinically useful non-nucleoside reverse transcriptase inhibitors

Rilpivirine is a second-generation diarylpyrimidine NNRTI that has found increasing utility in ART naïve HIV positive populations (36). After oral administration, it reaches peak plasma concentration in about 45- hours with a prolonged terminal half-life of 35-55 hours (37). Recent studies including report by Lamorde et al in Ugandan patients have demonstrated a definite food effect on the PK of this drug (38). Concomitant administration with low to moderate fat diet was shown in this study to result in enhanced serum level of the drug (38). Rilpivirine is metabolized

principally by *CYP3A4*, making it susceptible to significant drug interactions following co-administration with drugs that induce or inhibit activity of this enzyme (37, 38). This could affect the clearance of the drug with risk of virological failure. It is therefore advised to not be co-administered with avid *CYP3A4* inducing drug classes such as the Rifamycins [Rifampicin, Rifabutin] (37). Despite its recent incorporation into national and international treatment guidelines, a number of treatment emergent adverse effects have been reported in patients exposed to it. It has been shown to cause increase in serum creatinine without demonstrable change in measured eGFR (by formulas utilising non-creatinine based variables) (39). Rilpivirine has been suggested to inhibit multidrug anion transport organic cation transporter 2 (OCT2), thereby blocking the transport of creatinine from kidney capillaries into kidney tubular cells (39).

1.2.3 HIV Protease inhibitors

Since their discovery in 1996 (40), HIV protease inhibitors (PI's) have form a crucial part of most ART treatment regimen (18). They essentially target viral maturation by competitive inhibition of HIV protease enzyme (aspartyl protease) that plays a crucial role in cleavage of proteolytic precursors (Gag and Pol polyproteins) into key structural viral proteins (40). Consequently, this results in inability to combine these HIV viral particles into mature HIV virus (40). Despite early assurances regarding their efficacy and safety, a number of factors have limited the use of some of them as combination ART (cART). These include dose-dependent drug toxicities (41), relatively short half-life (requiring increasing dosing and high pill burden), and food interaction potentially impacting on their *AUC* with risk of virological failure (41), (42, 43). PI's are highly bound to plasma proteins, with 99% of Ritonavir (RTV) (41) and 86% of Atazanavir (ATV) bound to plasma proteins (41, 42). Ritonavir (as a

booster), Darunavir, Atazanavir, and boosted Lopinavir (in resource-limited settings) are currently the most widely used PI's in the HIV/AIDS therapeutics (41). Owing to factors highlighted above (including dose related adverse effects), RTV in particular is currently limited to use only as a booster for increasing serum concentration of concomitantly administered PI's or other drugs (41, 43). It is a powerful inhibitor of *CYP3A4* (a ubiquitous and highly polymorphic enzyme that metabolizes PI's and a significant number of other drugs) (41). This subsequently results in increased (boosted) serum concentration of the co-administered drugs (41). The exploitation of this pharmacogenetic interaction in ensuring favorable virological outcomes unfortunately contributes significantly to some of the clinical phenotype of PI-related inadvertent treatment-limiting adverse effects. The range of PI related adverse effects are protean, but amongst the most widely reported toxicity syndromes includes, interstitial nephritis, Indinavir (IDV) induced hyperbilirubinemia, and dyslipidemias (44). Unlike the uniformity of their primary mechanism of action (i.e. Inhibition of cleavage of key structural proteins), the mechanism of PI related adverse effects is diverse. These range from inhibition of renal tubular transport proteins (such as *OAT1*, *OAT3*, *MRP3*, *MATE*) by RTV; to decrease solubility of ATV in urine (especially in setting of alkaline pH) resulting in interstitial nephritis; as well as significant pharmacogenetic interaction highlighted earlier (44, 45).

1.2.4 Entry inhibitors

HIV-1 primarily utilizes CD4 cell surface receptors as primary targets for entry into immune competent cells. Additionally, it also exploits a complex of other cell surface chemokine co-receptors, including C-C chemokine receptor type 5 (*CCR5*), and C-X-C chemokine receptor type 4 (*CXCR4*) amongst others (46). This leads to release of HIV-1 viral core into the cytosol, and subsequent downstream processes

leading to viral replication (46). Fusion or entry inhibitors target and block the binding of HIV *gp120* envelope to *CCR5/CXCR4* chemokine receptors by allosterically inducing a conformational change in these receptors (46). Consequent upon this, HIV-1 viruses are therefore classified into *CCR5* trophic (R5-trophic) or *CXCR4* trophic (R4 trophic) viruses depending on whether they utilize the corresponding chemokine co-receptors to gain entry into immune competent cells. A tropism test to ascertain specific viral chemokine co-receptor preference is often advised before initiation of therapy with entry inhibitors (47). Amongst clinically characterized entry inhibitors includes Maraviroc (MVC) and Cenicriviroc (CVC). The latter is a dual *CCR2/CCR5* inhibitor (48). Maraviroc (figure 1.6) is a potent, selective, *CCR5* entry inhibitor, which has demonstrated excellent efficacy and safety profile in a number of clinical trials (49, 50). It is principally metabolized by *CYP3A4* and therefore vulnerable to drug interactions, resulting in significant changes to its *AUC* and risk of therapeutic failure. Advisedly, its dose for example should be increased from 300mg twice daily to 600mg twice daily when co-administered with EFV, due to the significant induction of its metabolism by EFV (51). MVC is increasingly evolving as a once daily elegant option for a cohort of HIV positive patients requiring a Nucleoside-sparing regimen (52). What has limited its utilization in this regard has been lack of robust pharmacokinetic data for such combination (53). A recent seminal report by Mora-Peris et al observed no short-term efficacy/safety concerns for a once daily nucleoside-sparing regimen comprised of 800mg/100mg/150 mg of Darunavir/Ritonavir/Maraviroc respectively (54). It is noteworthy however that this study was limited by its relatively small sample size (*N* = 11). Caution and closer monitoring is advised when switching to MVC especially from EFV based regimen, owing to risk of reduced MVC serum concentration (from

CYP3A4 induction by EFV) (55) and consequent risk of therapeutic failure. This effect may be sustained for several days because of long half-life of EFV (40-55 hours). Although clinical experience with MVC is still accruing, a range of adverse effects has been associated with it. These include upper respiratory tract infections, fever, rash, hepatotoxicity and reactivation of herpes infection (54, 55). Despite concerted attempts thus far, no specific *CXCR4* inhibitor has been found to be useful in HIV pharmacotherapy.

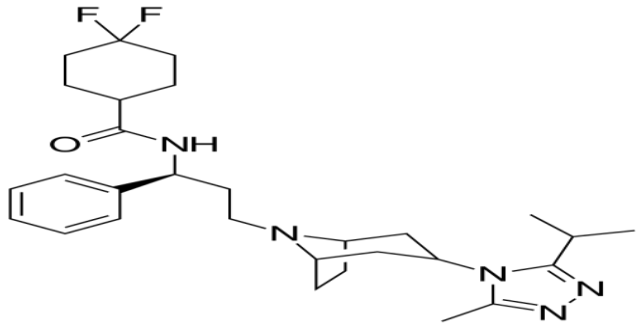


Figure 1.6: Molecular structure of Maraviroc

1.2.5 Integrase strand transfer inhibitors (InSTI's)

Raltegravir and Elvitegravir were the first to be marketed amongst this class of novel ART agents in both ART naïve and treatment experienced HIV positive patients (56, 57). It's second in class (Dolutegravir) received market authorisation (MA) in 2013 (58). They act principally by targeting *Integrase*, the key enzyme that catalyses *3'* end processing as well as DNA and strand transfer (59). This selective effect on strand transfer has been further clarified to involve an initial selective binding of *Integrase* to DNA-strand complex, and subsequent interaction with the two critical magnesium ions cofactors on both of the viral DNA/enzymes active sites (60). This dual but separate interaction with the *Integrase* enzyme, and the DNA of the virus makes the InSTI's unique amongst other classes of ART drugs. There remains uncertainty regarding the absolute bioavailability of Raltegravir owing to its variable

PK, but maximal serum concentration is observed after approximately 3 hours with a terminal half-life of about 9 hours (61). It requires twice daily dosing (62), with concomitant food ingestion significantly reducing *AUC* of the drug. Brainard et al (61) reported a reduction in its *AUC* by as much as 46% in patients on fatty meals (62). Various reports have suggested *uridine diphosphate glucoronyltransferase 1A1 (UGT1A1*) as the key enzyme involved in Raltegravir metabolism (63). *UGTIA1* also play a major role in metabolism of Dolutegravir with limited additional metabolism by *CYP3A4* (58). SNPs of both *UGT1A1, and CYP3A4* have thus far been shown to have significant impact on *AUC* of Raltegravir. This effect is less so but still significant for Dolutegravir (58).

Both Raltegravir and Dolutegravir have been shown to be safe and efficacious in a number of systematic studies involving various HIV cohorts including ART naïve, and treatment experienced patients (58, 62). The lack of comparable biological human homolog to the strand transfer step catalysed by HIV virus may account for some of the excellent tolerability of InSTI's, as well as their limited adverse effects profile. Both Dolutegravir (64), and Cobicistat-boosted-Elvitegravir (65) have been associated with increase in serum creatinine with no effect in intrinsic kidney function. Dolutegravir and Cobicistat (COBI) have been shown to be non-pathological inhibitors of the organic cation transporter 2 (OCT2), which is involved in uptake of creatinine from the kidney tubular capillaries into the tubule cells and subsequently secreted into the tubular lumen (65, 64, 66). This results in elevation of serum creatinine and decrease in creatinine clearance without change in true eGFR. Additionally, COBI is a well-established inhibitor of multidrug and toxin extruder protein 1 (MATE1). This transporter is located at the apical membrane, and is

responsible for efflux of creatinine and other substrates from the proximal tubular cells into the tubular lumen and urine (67).

1.3.0 Epidemiological perspectives of ART drug related adverse effects

Reporting of ART related drug toxicities are influenced by patient's socio-economic status, including study setting (observational or systematic studies), adverse events adjudication tools employed amongst other factors (68). These and other factors beyond the scope of this thesis have been suggested to account for marked variability in reported prevalence rates of ART drug related ADR's (68). Even amongst systematic studies, reported prevalence rates of ART related drug toxicities show marked variability (69). Despite guideline recommendation for grading and reporting of ADRs (70), this variability in reporting and lack of standardization have persisted (70). This has been attributed to a number of factors including differing sponsor identities, and non-uniformity of study outcome measures employed (70). Current adjudication algorithms for ADR assessment and interpretation in the general population derived primarily from the seminal work of Naranjo et al (71), who pioneered the Naranjo ADR probability scale. Proven efficacy and safety in randomised controlled clinical trials have always served as the standard for selection of various ART drugs into guideline recommended treatment regimen (18). Adverse effects reported from these studies and the controlled populations they study therefore represent an important component of the adverse events case burden of these drugs. It is however pertinent to note that post-marketing surveys (phase IV) captures other components of adverse effects morbidity not reported or limited by the relatively short observational period of systematic studies. Despite the inclusion of drug adverse effects reporting in the consolidated standards of reporting trials (CONSORT) statement (72), inadequate and partial reporting has been observed to

persist in a number of disease morbidities including HIV/AIDS. Consequent upon this, an extension of the CONSORT statement (73) was formulated specifically addressing issues with reporting of drug related adverse events (AE's). How this change has impacted on overall reporting quality remains unclear. In an attempt to further clarify on these recommendations and improve on ADR reporting in HIV patients recruited into clinical trials, Carr et al (74) suggested a number of key monitoring algorithms for improvement of AEs reporting in these studies. Recent prospective studies have adopted some of these recommendations, but the overall impact of this is still unknown. Additionally, study design appears (by systematic bias) to influence the reporting of ADR's. Randomised industry funded controlled clinical trials for example tended to report almost all spectrums of ADRs (including mild, moderate or severe), in contrast to non-profit studies that have emphasized on reporting serious adverse events only (69). The implication of this has been disproportionately higher burden of mild AE's, with severe AE's inappropriately reported with a lesser prevalence burden (69).

The patho-physiology of adverse reactions attributable to ART drugs are a legion. They include drug-drug interactions, subject cytokine profile, immune hyper activation, genetic susceptibility as well intrinsic physiological changes induced by putative components of the drugs (75). Metabolism of some ART drugs such as PI's by the ubiquitous and highly polymorphic cytochrome P450 enzymes systems makes drug-drug interactions an important contributor to the phenotype of PI related adverse events in patients exposed to them (41).

1.3.1.0 Factors impacting on prevalence and burden of ART drug related Toxicity

1.3.1.1 Gender

In the general population as in HIV positive patients on ART, gender has been reported as an important predisposing factor contributing to the development, severity, as well as the resulting clinical phenotypes of adverse reactions following exposure to these drugs (76, 77). Metanalysis of randomised controlled clinical trials of ART drugs in HIV patients including that by the food and drug administration (FDA) in the USA have failed to demonstrate any significant pharmacodynamic differences in response to these agents (78). However, despite the paucity of studies significantly powered *ab initio* to ascertain the role of gender in ART drug related adverse effects, data from both systematic and observational studies have reported higher prevalence rates of ART drug related ADRs in females than males (79). This is largely driven by data from studies exploring PK differences between males and females (80, 81). This is exhaustively summarised in table 1.1. Whilst the exact mechanism of gender in influencing the risk of ART related adverse effects remains unknown, total body weight, pharmacokinetics, and hormonal differences in men in comparison to women (amongst others) have been suggested as key determinants (81). Epidemiologically, there is likely to be underestimation of the exact prevalence rates of ART drug induced adverse events in women (82). This is so because until recently, women have represented relatively lower percentage of patients recruited into systematic studies to ascertain the efficacy and safety of ART drugs (82). In the AIDS Clinical Trials Group (ACTG) cohort of 11909 study participants for example, only 6.7% are women (82). Furthermore, despite changes to regulatory clinical trial

guidelines on gender enrolment into clinical trials, current enrolment of women into these studies still remains sub-optimal and has been estimated at about 20% (75).

It is noteworthy that PK of second generation NNRTI's such as Etravirine showed no significant gender related differences. Similarly, (perhaps largely driven by limited experience with these agents), no significant gender related PK differences have been reported with either InSTI, or entry inhibitors. The pharmaco-epidemiology of these agents however potentially could change as real-world experience with them becomes more established.

Table 1.1: Studies exploring the role of gender in pharmacokinetics of antiretroviral therapy (ART) drugs

Study	ART drug class	ART drug PK characteristics	Gender difference	Clinical toxicity phenotype	Comment
Anderson et al (83)	NRTI	Zidovudine (AZT) Lamivudine (3TC)	Significantly higher concentration of both AZT and 3TC levels of intracellular triphosphate in females (reported ratios of 2.3 and 1.3 for AZT and 3TC respectively).	Risk of AZT and 3TC adverse drug reactions (ADR's)	It is noteworthy that unlike other ART drugs, for NRTI's, intracellular phosphorylated triphosphate levels rather than plasma concentration correlates with risk of adverse effects.
Stretcher et al (84)	NRTI	AZT	Reported higher intracellular levels of AZT triphosphate in females		
Moore et al (85)	NRTI	Didanosine (DDI)	Higher incidence of DDI related ADR's in women. Additionally women have a higher likelihood of DDT adjustment or cessation of therapy than men		
Burger et al (86)	NNRTI's	Efavirenz (EFV) drug levels	60% Higher EFV serum levels than male cohorts (86)	Increased susceptibility to EFV toxicity and virological failure (87). Also higher risk of discontinuation of therapy due to toxicity (88)	Burger et al (86) reported EFV serum concentration of 4.0mg/L and 2.8mg/L in females and males respectively. Other studies including 2NN data support this finding (89)
La Porte et al (90)	NNRTI	Nevirapine (NVP)	Higher median NVP concentration (6.7mg/l) in females compared to males (5.5mg/l)		No conclusive sex related differences in the PK of NVP
Regazzi et al (91)		NVP clearance	44% higher *Cmax* in females compared to males		The consistently higher NVP serum concentration and lower clearance reported in women partly explains the disproportionately higher incidence of NVP ADR's in women compared to male counterparts.
Sanne et al (92)	NNRTI	NVP	Women with low BMI had a higher risk of hepatotoxicity compared with male counterparts	Combined risk of NVP hypersensitivity which manifest as either rash or hepatotoxicity or both	The risk of hepatotoxicity in Sanne et al's report was higher in women with higher CD4 count. This perhaps informs the suggestion by some treatment guidelines for NVP containing regimen not be commenced in women with

					CD4 count of >250 cells/mm^3.
Hodder et al (93)	NNRTI	Rilpivirine clearance	13.6% lower apparent clearance in females compared to males		
Burger et al (94)	Protease inhibitor (PI)	Boosted Lopinavir level (LPV/r)	Higher LPV and LPV ratio in females compared to male counterparts		Other PK studies including Ofotokun et al (95) failed to show any gender related difference in PK of LPV/r. it is probable that the lower body weight reported in Burger et al's study (94) may have accounted for this discordant outcomes
Fletcher et al (96)	PI	Saquinavir (SQV) serum concentration	Significantly higher median Saquinavir Cmin and AUC in females. Additionally, women had a lower weight-adjusted clearance of SQV		There was a larger proportion of females with suppressed RNA viral copies (<500cells/ml) in this study, perhaps a consequence of SQV serum concentration
Burger et al (97)	PI	Indinavir serum levels	No gender related difference in IDV levels (see comment)		However more women had IDV TDM assays because of concerns for drug toxicity. Additionally there were more adverse event related dose adjustments in females (9.7%) than males (1.1%)
Arasteh et al (98)	PI	Darunavir (DRV)	Population PK analyses from POWER 1 & 2 studies (n =68) showed that females had a higher AUC_{24h} for DRV compared to male patients		The difference in DRV AUC_{24h} in this report was not deemed to be clinically significant.
Currier et al (99)	PI	Ritonavir (RTV)	More incidence of RTV related neurological and gastroenterological adverse events in females (61%) than males (27%)		
Pai et al (100)	PI	SQV	Higher SVQ $AUC_{0\text{-}24H}$ in females compared	Potential for SQV adverse events	
Flexner et al (101)	PI	Atazanavir (ATV)	Higher mean serum concentration in women.		

1.3.1.2 Pharmacogenetics

Inter-individual variation in both efficacy and adverse events associated with ART drugs represents a significant morbidity of HIV patients on treatment (86). Whilst extraneous factors such as drug concordance and gender amongst others may contribute to this, pharmacogenetics increasingly has been identified as a significant determinant of this variation (102). Single nucleotide polymorphisms (SNPs) represent the most common genetic variation in the human genome (102). By definition they are referred to as nucleotide sequence variation in human DNA with an allele frequency of greater than 1% (103). This is to differentiate them from variation with allele frequency of less than 1%, often referred to as gene mutation. Since the groundbreaking work of the Human genome project and subsequent elucidation of the genetic code, increasing body of evidence links SNPs with various HIV comorbidity phenotypes and adverse drug toxicities (102). A significant proportion of these studies have largely utilised a candidate gene approach. Recently however, there has been increasing attempts at exploring relevant genotype-phenotype correlation using genome wide association studies in HIV positive patients exposed to ART drugs (104, 105). SNPs have been shown to occur about every 200-300 base pairs along the 3 billion letters of the genetic code (103). Specific genes regulate various proteins involved in the bio-disposition of ART drugs including their absorption, distribution, metabolism and elimination from the body. Variation in these genes represented as SNPs do impact on the bio-disposition of these drugs resulting in either reduced efficacy (including virological failure) due to decreased tissue exposure or clinical toxicity (103).

It is often difficult to conclusively associate a single SNP with a particular clinical phenotype or drug related adverse event. This is so because of the multiplicity of

other factors associated with such adverse event. However, where a cluster of SNPs called haplotypes consistently reports association with a particular ART adverse event, causality in this case is more robust and clear. Amongst the various ART related drug toxicities explored by pharmacogenetic studies includes hypersensitivity syndromes (106), kidney tubular dysfunction (107, 108, 109,110), liver injury, peripheral neuropathy, and various central nervous system related adverse effects amongst others (106). The ultimate goal is to utilize these data in identifying population at risk of developing these adverse events based on their possession of the relevant SNP thereby avoiding exposure to the incriminating ART drug in the first place. Unfortunately, the quality of studies reported thus far (with a few exceptions), and data generated from most pharmacogenetic studies exploring ART drug toxicities are not robust enough to allow their utilization as surrogate genetic markers or point of care assays yet. In a few reported cases what initially appears as significant associations between a particular SNP and a clinical ADR phenotype in candidate gene analysis doesn't prove significant in further genetic evaluation including GWAS. A pertinent example is the reported association between the possession of *UGT1A1* promoter (TA)n repeat (rs8175317), and risk of Atazanavir induced hyperbilirubinemia (111). Further exploration of this association in pharmacogenetic studies including correction for *UGT1A1* (rs8175317) carriage failed to replicate this finding (112). Table 1.2 summarizes key pharmacogenetic studies exploring ART drug exposure and various clinical drug toxicity phenotypes

Table 1.2: Summary of pharmacogenetic studies exploring association between anti-retroviral therapy (ART) drug exposure and risk of ART related adverse drug reactions

Study	**Study design**	**ART class**	**ART drug**	**Candidate gene/genotype/haplotype explored**	**Clinical ADR phenotype**	**Comment**
Mallal et al (106)	Case control	NRTI	Abacavir (ABC)	*HLA B*5701*	ABC hypersensitivity. People from Indian sub-continent have the highest carriage rates of this haplotype (15%)	This represent the first and perhaps the only candidate gene in the whole of HIV/AIDS therapeutics to be exploited in order to guide clinical therapy
Saag et al (113) for SHAPE trial	Randomised controlled trial (RCT)	NRTI	ABC	*HLA B*5701*	Risk of ABC hypersensitivity	Both SHAPE and PREDICT represent the first RCT's to established a clinical role for this genotype in the triage of HIV patients potentially at risk of ABC hypersensitivity
Mallal et al (114) for PREDICT trial group	RCT	NRTI	ABC	*HLA B*5701*	Risk of ABC hypersensitivity	
Izzedine et al (109)	Case control	NRTI	TDF	*ABCC2 1249 G>A* (rs2273697) *ABCC2 Haplotypes: CGAC and CATC*	Risk of Kidney tubular dysfunction (KTD)	Strong allelic link between *ABCC2 1249 G>A* and risk of KTD. Conversely *ABCC2* haplotypes *CGAC*, and *CATC* were found to be protective and increased risk of KTD respectively
Rodriquez-Novoa et al (107)	Case control	NRTI	TDF	*ABCC2 24CC* (rs717620; MRP2)	KTD	Possession of genotype *CC* at position 24 of the *ABCC2 gene* has seminally been reported by this study to be associated with increased risk of KTD
Nishijima et al (2012) (108)	Case control	NRTI	TDF	*ABCC2 24CC* (rs717620; MRP2)	KTD	
Pushpakom et al	Case control	NRTI	TDF	*ABCC10* (MRP7;	KTD	The first study to both ascertain MRP7 as a putative

(110)				rs2125739, rs9349256) *ABCC2-ABCC1O* extended haplotype (*GGC-CGTC*)		TDF transporter and link possession of intronic SNPs of *ABCC10* and their extended haplotype with risk of KTD
Nishijima et al 2015 (115)	Case control	NRTI	TDF	*ABCC2 24CC (rs717620; MRP2)*	Decrease in eGFR	Neither allele C or genotype CC were associated with risk of KTD
Likanonsakul 2015 (116)	Case control	NRTI	TDF	*ABCC2 -24CT 1249GA*	Urinary β2-microglobulin thresholds	No significant association with KTD
Hulgan et al (117)	Case control	NRTI	Didanosine (DDI)	*7028C>T, 10398G>A,* and *(118)68G>A* (within haplotype T of *mtDNA*)	Peripheral Neuropathy	
Canter et al (119)	Case control	NRTI	DDI	*4216C* and *4917G* (within haplotype T of *mtDNA*)	Peripheral neuropathy	This is irrespective of age and HIV RNA viral load levels amongst other co-variates
Felley et al(28)	Case control	NRTI	DDI	*CFTR mutations and SPINK-1 Polymorphisms*	Suggested association with risk of hyperamylasemia and pancreatitis	Further studies still awaited to ascertain these findings
Holzinger et al (120)	Case control	NNRTI	Efavirenz (EFV)	*CYP2B6 516G>T (rs3745274)*	Associated with high EFV serum concentration.	Potentially increased risk of CNS adverse effects. Increased frequency of this allele in patients of African ancestry
Wyen et al (121)	Case control	NNRTI	EFV	*CYP2B6 983 T>C* (rs28399499)	Increased EFV serum levels	
Saitoh et al (122)	Case control	NNRTI	Nevirapine (NVP)	*CYP2B6 516G>T*	Increased NVP *AUC*	Potential for risk of NVP ADRs including rash and liver toxicity Associated with
Carr et al (123)	Case control	NNRTI	NVP	*HLA-C*04: 01*	Increased risk of Steven-Johnson syndrome	This study was carried out in Malawian patient cohort
Martin et al (124)	Case control	NNRTI	NVP	*HLA-DRB1*01:01*	High CD4 count and	Predominantly Australian patient cohort

					increased risk of Liver injury and skin rash	
Gatanaga et al (125)	Case control	NNRTI	NVP	*HLACw*08*	Risk of NVP rash	Japanese cohort
Ritchie et al (126)	Case control	NNRTI	NVP	*MDR1 3435 C>T*	Protective of risk of NVP drug related liver injury (DILI)	
Haas et al (127)	RCT	NNRTI	NVP	*MDR1 3435 C>T*	Reduced risk of NVP related DILI	First RCT to ascertain protective effect of this genotype in a clinical trial setting
Rodriquez-Novoa et al (128), Rotger et al (129)	Case control	PI	Atazanavir (ATV)	*UGT1A1 (*6*6, *7*7, *6*7)*	Increased risk of hyperbilirubinemia with possession of these genotypes	This association was consistent across diverse ethnic populations.
Rodriquez-Novoa et al (128)	Case control	PI	ATV	*MDR1 3435 C/C*	Increased risk of ATV induced hyperbilirubinemia with possession of this P-glycoprotein genotype	
Fauvel et al (130)	Prospective cohort	PI-based regimen		SNPs of *APOC3*	Increased risk of dyslipidaemias	
Foulkes et al (131)	Prospective cohort	PI-based regimen		*APOC3* and *APOA1* genotypes	Risk of hyperlipidaemias	
Tarr et al (132)	Prospective cohort (Swiss HIV cohort data)	PI-based regimen		SNPs of *APOC3* and *APOE*	Patients with all 3 SNPs of *APOC3* and *APOE* were associated with increased risk of hypertriglyceridemia	This study also suggested association between these studied SNPs and risk of lipodystrophy

1.3.1.3 Ethnicity

There have been notable, well-established racial differences in PK (133,134), PD (134, 118), and pharmacogenetic (134, 118) profiles of patients from diverse study settings. Unfortunately, this is not without morbidity, as it has increasingly been shown to impact adversely and contribute to the burden of ART drug related toxicities (135). Amongst the most extensively studied race-related ART drug toxicity has been those exploring EFV (86) and NVP in varied ethnic populations. The PK of EFV is associated with extensive inter-individual variability (133, 118). This has been shown to result in downstream effects including treatment failure (sub-therapeutic levels) with concentration less than1μg/ml, and CNS toxicity when serum concentrations exceed 4μg/ml (136, 137, 138). Ethnicity amongst other factors including body weight, and concomitantly administered drugs (such as Rifampicin), significantly impact on EFV serum concentration (133). In an earlier report, Stohr et al for example showed that black ethnicity was significantly associated with about 59% higher EFV serum concentration than comparable cohorts (133), with a potential concern for risk of EFV associated CNS adverse effects. EFV as highlighted earlier is metabolised predominantly by *CYP2B6* and to a limited extent by *CYP3A4* (34). Studies of polymorphisms of these enzymes vis-à-vis EFV adverse effects in various ethnic populations have reported varying risk profile. Carriage of the genotype *CYP2B6 516G>T* for example has consistently been shown to correlate with higher EFV concentrations across a broad range of ethnic populations (139). A number of studies (124, 140) have also recently explored the pharmacogenetic underpinning behind cutaneous hypersensitivity syndromes/liver injury following exposure to NVP in diverse ethnic populations. NVP rash is a well-established treatment limiting AE reported in a wide cohort of patients with an overall incidence

estimated to be about 2.8 times higher in Thai than comparable white patient cohorts (141). In a study exploring the role of environmental and genetic factors in the pathogenesis of NVP hypersensitivity in Malawians, Carr et al reported association between *HLA-C*04: 01* carriage and development of Stevens-Johnson syndrome in HIV infected Malawian patients exposed to NVP (123). Earlier report in predominantly white Australian population have suggested a causal link between the *HLA-DRB1*01:01* genotype, higher CD4 T-cell, and risk of liver/skin rash in cohort of patients exposed to NVP (124). Conversely, studies in Japanese patients reported more of association with *HLACw*08* genotype (125) than other previously suggested genotypes. A recurrent theme of reports discussed thus far has been the consistent phenotype-genotype linkage in specific ethnic populations. This perhaps helps explain the defining role of genetics as one amongst a number of determinants of ART drug induced ADR in various ethnic populations.

What has limited the clinical utility of the afore-mentioned surrogate genetic markers here and elsewhere in this thesis as point of care assays to date, includes limited sample size of these studies and their relatively low predictive values amongst others.

1.4.0 Specific antiretroviral therapy (ART) drug related toxicity syndromes

1.4.1 Tenofovir induced Kidney tubular dysfunction (KTD)

Tenofovir either given as one of its available salts i.e. Tenofovir disoproxil fumarate (TDF), or Tenofovir alafenamide fumarate (TAF) is a pro-drug which undergoes rapid bio-activation to its active moiety Tenofovir (TDF) with a terminal half-life of about 30 hours (142) (figure 1.8). It is metabolized and cleared from plasma through a combination of glomerular filtration and active secretion from kidney tubules (143). TDF is the most widely used of the NRTI's in HIV therapeutics, largely due to

its well-established efficacy and safety profile from a number of studies (144, 145). It has however continued to generate concerns owing to its propensity to cause kidney injury in HIV positive patients exposed to it (145). The EUROSIDA investigators for example reported that for every 10 year exposure to TDF, there was about 16% risk of decline in eGFR, with the magnitude of decline highest in patients on ATV/r regimen (22%) compared to other PI based regimen (8%, and 11% for LPV/r and Indinavir respectively) (146). Contemporaneous use of both PI/TDF regiment was associated with a faster rate of decline (41%) in kidney function in the study (146). Despite this case burden, the exact pattern (clinical phenotype), diagnostic markers, and mechanism of kidney involvement is still uncertain. Kidney tubular dysfunction and Fanconi syndromes were amongst the most widely studied and reported clinical and laboratory phenotypes from both longitudinal and systematic studies (144, 145). The reported prevalence of clinically significant TDF related kidney injury is variable, but it is estimated to range between 2-11% (147,148, 149). Organic Influx transporters such as organic anion transporter 1 (OAT1) encoded by *SLC22A6,* and OAT3 (*SLC22A8*) located at the basolateral membranes are suggested to mediate the entry of TDF into kidney tubular cells (KTC). Efflux transporters such as multi-resistant protein 4 (MRP4, encoded by *ABCC4*), and MRP7 (encoded by *ABCC10*) located on apical membrane are involved in secretion of TDF from KTC into the tubular lumen (110, 150, 151) (figure 1.7). Both transporters have been reported to be polymorphic, with single nucleotide polymorphisms (SNP) of genes encoding them (particularly MRP2 (*ABCC2*), and MRP7 (*ABCC10*) proposed to confer increased risk of KTD in HIV positive patient exposed to TDF (107, 110).

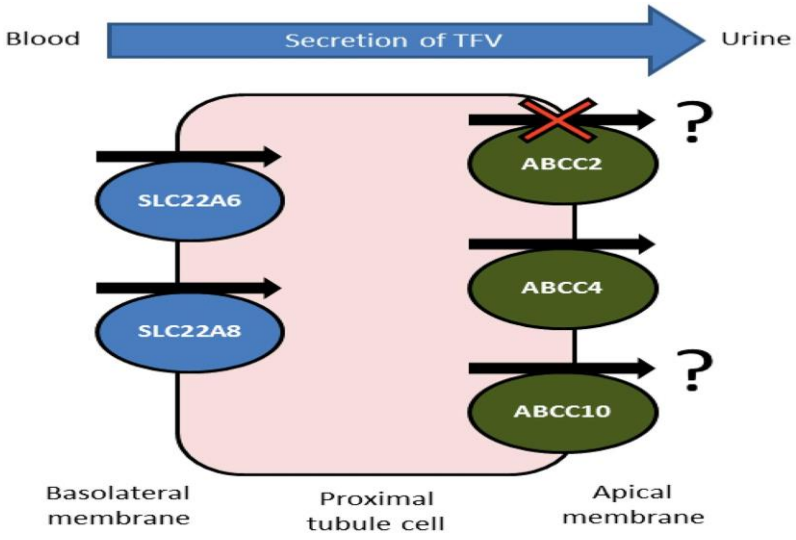


Figure 1.7: Relative disposition and kinetics of known Tenofovir Kidney transporters [adapted from Moss DM et al] (152)

Recently, three studies with different study methodology, patient populations, and surrogate markers of kidney injury, established associations between polymorphisms of *ABCC2* genes (MRP2, rs717620), and risk of TDF related KTD (107, 108, 109). In a seminal report, Izzedine et al showed a strong allelic association between carriage of the *CATC* haplotype (represents combination of SNPs at position 1249, 3563, 24, and 3972 within the *ABCC2* gene) and risk of KTD (OR 6.11 [95% CI, 1.19–31.15]; P <0.02). Conversely, the possession of the *CGAC* haplotype in this report was found to confer a protective effect on risk of kidney injury through a higher TDF secretion from the PCTs (109). All three reports were limited by their retrospective case control designs. This is so because early onset KTD may have been missed by these analyses and this limits ability to establish causality. In another report, Rodriquez-Novoa et al in pharmacogenetic analyses of the Liverpool therapeutic drug monitoring (TDM) registry data, reported association between possession of genotype *CC* at position 24 of *ABCC2* gene (rs717620), and risk of TDF-induced KTD in multivariate analysis (107). Older age, and low body weight

were additional factors found in this study to be associated with an increased risk of KTD in cohorts of HIV positive patients exposed to TDF (107). Conversely, a recent pharmacogenetic study (115) in a prospective cohort of HIV positive patients failed to establish association between the *ABCC2 24CC* (rs717620) SNP, and risk of KTD. This further highlights the lack of agreement regarding the relationship between possession of this SNP (*ABCC2 24CC*), and risk of KTD in these cohorts of patients. Nevertheless, the findings from previously highlighted reports (linking increased KTD risk with *ABCC2_24CC* genotype) (107, 108, 109) presented an unresolved mechanistic paradox. Studies thus far have failed to associate MRP2 (*ABCC2*) with transport of TDF, rather mechanistic reports thus far have consistently associated MRP4 (*ABCC4*), and MRP7 (*ABCC10*) as putative TDF transporters (110, 150, 151). How possession of *ABCC2 24CC* genotype mediates kidney tubular dysfunction therefore remains uncertain. Suggested mechanisms for this paradox includes decreased secretion of TDF by *ABCC2-24CC* homozygotes; transport by MRP2 of yet to be identified factor that influences TDF toxicity on the kidneys; also proposed is the suggestion that *ABCC2-24CC* might be in linkage disequilibrium with yet to be identified SNPs that influence TDF secretion by the kidneys (107)

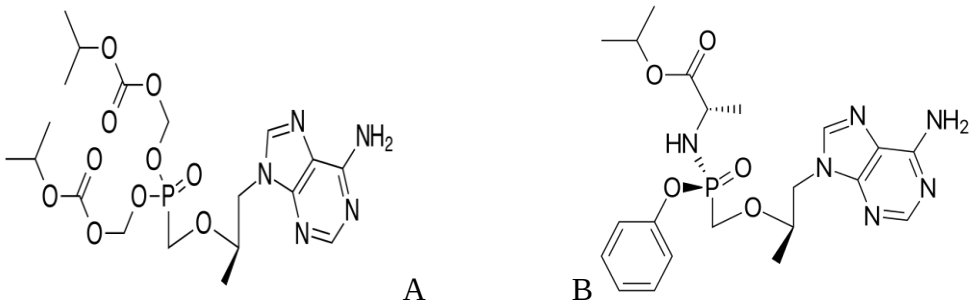


Figure 1.8: Molecular Structure of Tenofovir disoproxil fumarate (A), and Tenofovir alafenamide fumarate (B)

TDF is a well-established substrate for MRP4 (*ABCC4*). Pharmacogenetic exploration of the various SNPs and Haplotypes of *ABCC4* have thus far only reported conflicting data regarding the risk of KTD with any specific SNP or haplotype associated with this gene. The *ABCC4 3463G* SNP for example has been associated with decreased kidney clearance of TDF (153). In his seminal report, Izzedine et al (109) first reported allelic association between the possession of the *ABCC4 669C>T* genotype and risk of KTD. Subsequent pharmacogenetic reports including those by Nishijima et al (108) and Rodriguez-Novoa et al (107) failed to establish this association. How differences in the study methodology, patient populations, and surrogate markers of kidney dysfunction deployed explain these contradictory findings is still open to debate.

In a recent mechanistic/pharmacogenetic report, Pushpakom et al (110) both established MRP7 (*ABCC10*) as a TDF transporter and subsequently demonstrated increased risk of KTD with possession of two *ABCC10* SNPs (MRP7, rs2125739, rs9349256) and their haplotypes (OR 2.1, P = 0.05) in HIV positive patients. In this study, possession of the *ABCC10* SNP (rs9349256) was also associated with increased urinary phosphate excretion (a surrogate marker of TDF induced kidney tubular dysfunction) (110).

P-glycoprotein (PGP) has been shown to influence the absorption of TDF through the enterocytes but has no effect on its systemic kinetics including its kidney transport (154). Pharmacogenetic studies so far have failed to demonstrate any association between the polymorphisms in P-glycoprotein *MDR-1* gene (including1236 C>T, 2677G>T/A, and 3435C>T) and risk of TDF-induced KTD (107, 109).

Recently, a new formulation of Tenofovir called Tenofovir alafenamide fumarate (TAF) is generating intense mechanistic interest (155). Its absorption across intestinal epithelium as TAF is influenced by p-glycoprotein. It circulates within the blood steam as, and subsequent transport into cytosol of immune competent cells as TAF (156). Intracellularly, it is converted by cellular kinases into Tenofovir diphosphate (156). Its preferentially high intracellular disposition (in form of Tenofovir-diphosphate), and relatively limited plasma concentration meant there is reduced systemic exposure to this drug. The implication of these salutary properties is a probable reduction in risk of kidney related adverse effects. Indeed a recent seminal study have reported reduced incidence of KTD (as evidenced by hypophosphatemia and proteinuria), as well as bone mineral density when TAF was part of a regimen comprised of Cobicistat (COBI), Elvitegravir (EVG), Emtricitabine (FTC) (157). Additional reports including the recent 48-week follow-up switch data from Pozniak et al, have suggested increased renal safety with regimen consisting of TAF in patients with mild to moderate kidney impairment (158). As more studies continue to explore the pattern and definitive clinical phenotype of TDF-related kidney injury, emphasis remains on current point of care measures aimed at reducing this risk. These includes, close monitoring of kidney function with conventional markers of kidney injury in HIV positive patients commencing the drug, avoidance of TDF in HIV patients with established chronic kidney disease (CKD) amongst other measures.

1.5.0 Adjudication of ART drug related Kidney adverse effects

1.5.1 Determination of kidney dysfunction

As in the general population, the exact burden of ART drug related adverse effects is very difficult to established (80). This has been attributed to a number of factors described in other sections of this thesis. These include lack of uniformity of reporting methods and algorithms, differences in patient's population, variable ADR adjudication process, and marked discrepancy in monitoring process (68, 69). The lack of agreement in the accepted surrogate markers of kidney dysfunction for example has contributed significantly to uncertainty regarding the prevalence and pattern of TDF related kidney tubular dysfunction. Whilst both observational and clinical trial data have suggested varying degrees of kidney dysfunction, traditional markers of kidney injury such as eGFR and urinary ACR/PCR have so far been suggested to miss early/sub-clinical forms of TDF related kidney injury (147, 149). Consequently, having a biomarker reporting early/subclinical forms of ART drug related injury will be important as it has the potential to reduce morbidity associated with these drugs and serve as an effective monitoring tool. Recently, some low molecular weight proteinuria (LMWP) such as kidney injury molecule 1 (KIM-1), N-acetyl-beta-D-Glucosaminidase (NAG), neutrophil gelatinase associated lipocalin (NGAL), retinol binding protein (RBP), interleukin-18 (IL-18), and L-type fatty acid binding protein (L-FABP) have increasing been shown to correlate with sub-clinical forms of various ART drug related kidney injury (159, 160, 161, 162). Whether such associations are consistent enough to allow their use as diagnostic/monitoring surrogate markers is still unknown.

1.5.1.1 Kidney injury molecule 1 (KIM-1)

KIM-1 (kidney injury molecule 1) is a putative, inducible, type-I trans-membrane tubular adhesion glycoprotein facultatively expressed on the surface of kidney tubular cells (163). Its trans-membrane domain undergoes proximal membrane cleavage, releasing the ectodermal KIM-1 that is excreted and quantifiable in urine (163). It is not usually expressed in normal kidney tubular cells. However, in response to a variety of insults including ischemic reperfusion injury and metabolic/infective stress, its expression is markedly increased (163) where it functions as a scavenger receptor (163). Recent reports from a variety of experimental and observational studies have suggested KIM-1 as a potential early surrogate marker of kidney tubular injury (159, 160) (figure 1.9). In a seminal report determining its diagnostic utility in Humans, Han et al reported high levels of normalised urinary KIM-1 in patients with clinically adjudicated acute kidney injury (AKI) (164). In this report, one unit rise in normalized KIM-1 levels was associated with a twelve fold increased odds of ischaemic acute tubular necrosis (164). Other subsequent studies explored the relationships between elevated KIM-1 levels and actuarial kidney survival. In the Multi-Ethnic Study of Atherosclerosis (MESA) for example, elevated KIM-1 and IL-8 values were significantly associated with increased risk of kidney function decline independent of urine albumin or eGFR (165). Furthermore, other studies associate higher levels of KIM-1 with increased mortality risk or need for dialysis in AKI patients presenting to accident and emergency departments (166). However, there is limited data pertaining to its use in assessment of kidney function in HIV positive patients on ART drugs. In a recent report by Peralta et al in HIV positive patients, higher KIM-1 values were associated with doubling of mortality risk in demographically adjusted model. This effect was

however completely attenuated and found to be insignificant with full adjustment (167). Despite the multiplicity of biomarkers so far suggested to predict or detect kidney injury earlier than traditional assays (eGFR, urine PCR/ACR), KIM-1 data from a wide variety of sources have supported its potential role as tubular biomarker following exposure to ART drugs.

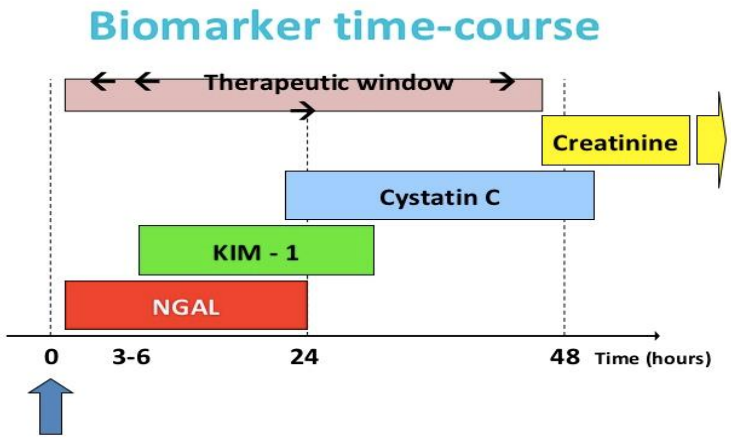


Figure 1.9: A schematic representation of time course of standard and novel kidney biomarkers [Adapted from Mcllroy DR et al] (168)

1.5.1.2 Retinol Binding Protein (RBP)

This is a low molecular weight single polypeptide produced by liver as well fat cells. It primarily transports retinol (vitamin A) in plasma and it is freely filtered, and completely reabsorbed by kidney tubules (169). This, in addition to pH stability of RBP, as well as its ease and reliability of measurements has suggested a potential role for it as a surrogate marker of kidney tubular dysfunction (169). It is expressed as RBPCR, corrected for urinary creatinine excretion (161). In a cross-sectional study exploring the diagnostic utility of low molecular weight proteinuria (LMWP), Hall et al reported a higher normalised urinary RBP in cohorts of patients on TDF

based regimen (161). This study explored the diagnostic utility of a number of LMWP including RBP, NAG, and normalised urine albumin (urine ACR) in HIV positive patients on various ART regimens (161). NAG levels were elevated in all cohorts of patients regardless of ART exposure whereas normalised urinary RBP levels were only raised in patients on TDF based regimen. Inhibitory effect of TDF on tubular transport of LMWP including RBP has been suggested to account for this increased urinary kinetics (161). Other reports whilst exploring the same diagnostic utility of RBP in HIV patients on ART, reported a higher proportion of tubular proteinuria in these cohorts of patients (162). Notably, those with no overt evidence of kidney disease had normalised urine RBP within the reference range. This has been suggested to underlie a potential role for RBP as surrogate marker of kidney function in these cohorts of patients (162). In a recent mechanistic study, Calcagno et al reported significant association between urinary TDF concentration and urinary RBP levels (170). It is pertinent to note that the relationship between serum Tenofovir concentration and risk of kidney dysfunction have remained contentious. Previous studies including report by Ray et al have failed to establish any significant association between TDF concentrations and risk of kidney injury (171). Establishing RBP as a diagnostic marker of clinical, and sub-clinical injury in HIV positive patients on ART drugs will contribute immensely in ascertaining both the prevalence as well as the clinical phenotypes of some these toxicities.

1.5.1.3 Neutrophil gelatinase associated lipocalin (NGAL)

These are diverse groups of proteins called lipocalins, the hallmark of which is the possession of a three-dimensional structure as a unifying characteristic (172). They are comprised of a 178-amino acid (25-kilo Dalton) glycoprotein expressed by neutrophils and most epithelial cells including kidney tubular cells (172). Their exact

function is still unclear, but they are suggested to be involved in kidney tubular regeneration (172). NGAL expression by kidney tubular cells has been shown to increase following exposure to metabolic, inflammatory, and infective stress (173). NGAL as a prospective kidney injury marker has been extensively studied in varied clinical settings in both HIV cohorts and the general population (173). Paragas et al working on transgenic mouse model reported a higher expression of NGAL in kidney biopsies of HIV positive patients with HIVAN compared to those other kidney morbidities (174). Following on from this, both diagnostic and prognostic kidney biomarker roles has been suggested for NGAL by a number of studies including metanalysis of these studies by Hasse et al (175). In a recent study evaluating LMWP in a hospital-based cohort, Campbell et al reported 67% of studied participants having NGALCR (corrected for urinary creatinine excretion) above diagnostic thresholds published by NGAL manufacturers (162). NGAL's ability to correlate and track early kidney tubular injury in both the general population and limited HIV cohorts on ART, as well as its ease of laboratory measurement at point-of-care potentially could suggest a role for it as a surrogate marker of KTD in these cohorts of patients on ART. The totality of available data thus far remains insufficient to suggest application of NGAL as point of care assay in HIV positive patients on ART.

1.5.1.4 N-acetyl-β-D-glucosaminidase (NAG)

This is a 150kDa glycolytic lysosomal protein expressed by tubular epithelial cells as well as in other diverse tissues (176). It is widely distributed amongst kidney tubular cells (176, 177), with increased expression and urinary excretion following exposure to infective, metabolic and ischaemic stress (177). In an evaluation of prevalence of sub-clinical kidney tubular injury in a cohort of HIV patients on various ART

regimens, Hall et al reported higher urinary levels of NAG/C (corrected for urinary creatinine excretion) in patients on TDF based regimen compared to those on alternative regimen (161). In spite of this, NAG/C in common with other LMWP evaluated in this study (including RBPCR) failed to detect significant degrees of sub-clinical kidney tubular dysfunction reported. The multifactorial nature of ART related kidney morbidities suggest that multiple biomarkers utilised as a cluster, may be required to robustly report clinical and subclinical degrees of kidney injury following exposure to these drugs. Indeed a recent report by scherzer et al, showed that NAG, KIM-1, and alpha-1 microglobulin utilised as a cluster were as effective as an 8-panel biomarker in predicting incident chronic kidney disease in HIV infected women (178). As with other novel biomarkers, NAG is still undergoing further clinical evaluation to prospectively determine its role as diagnostic surrogate marker of early kidney tubular dysfunction in HIV positive patients exposed to ART drugs.

1.5.1.5 L type-fatty acid binding protein (L-FABP)

This is usually found in the cytosol of proximal tubular cells, where it primary binds and transports fatty acids into the mitochondria for subsequent β-oxidation as part of the body's energy generation pathway (179). A number of reports from the general population have suggested its potential utility as an early marker of kidney tubular dysfunction in varied clinical settings including patients with acute kidney injury and solid organ transplant cohorts (167). In HIV population, it has been shown to be markedly elevated in patients on EFV compared with healthy controls (180). In further characterisation of probable diagnostic role of L-FABP, Peralta et al studied association of L-FABP (amongst other markers such as urine ACR, KIM-1, NGAL, IL-18) with a 10-year all-cause mortality risk in a cohort of 908 HIV positive women

(180). There was a J-shaped relationship between L-FABP and mortality, with IL-18 showing the most consistent relationship with mortality in this study. These associations were independent of either thresholds of urine ACR, or eGFRcys (eGFR corrected for Cystatin C excretion used as a surrogate marker of kidney dysfunction) in this study (167). Whilst a possible inference from these reports is potential utility of L-FABP to identify early kidney injury in these cohorts of patients following ART exposure, there is need for more robust prospective data to validate its use in this setting.

CHAPTER 2

SYSTEMATIC REVIEW AND METANALYSIS OF GENETIC DETERMINANTS OF TENOFOVIR INDUCED KIDNEY INJURY IN HIV POSITIVE PATIENTS

2.0 Background

2.1.1 Tenofovir and kidney disease

Over the past few years, there has been an increasing interest in the role of genetics in the pathogenesis of TDF induced kidney tubular dysfunction (KTD) (107, 108, 110). Comprehensive narrative review of the role of SNP's encoding proteins involved the biodisposition of TDF, and risk of Kidney injury is provided in chapter one of this thesis. However, despite these reported associations, it remains uncertain if the variability associated with these polymorphisms is robust and significant enough to recommend them as point of care assays or reliable risk stratification tools. In this chapter, we have attempted for the first time to systematically analyse current pharmacogenetic data with regards to the reported SNPs to in order to ascertain the strength of association with risk for KTD in HIV positive patients exposed to TDF.

2.1.2 Aim of systematic review

This is to attempt a systematic review of available pharmacogenetic data (including SNP's of *ABCC2, ABCC4, ABCC10, and ABCB1* genes*)* linking TDF exposure with risk of kidney injury from a wide variety of sources. This was in order to ascertain any degree of agreement between them, as well as consistency, and robustness of such association.

2.2.0 Materials and Method

2.2.1 Search strategy

We adopted a broad search strategy (encompassing all languages, all studies, and available years (up to 30/08/2016). We did a comprehensive search of pre-specified databases including PUBMED, MEDLINE, Cochrane database of systematic reviews, Web of Science, (dbSNP), *PharmGKB*, clinical trials.org, CROI, and

EMBASE to identify studies using the following medical subject heading (MeSH) terms: ((“Tenofovir Disoproxil Fumarate”[MeSH Terms] OR “Tenofovir”[All Fields] OR “TDF”[MeSH Terms]) OR “TDF”[MeSH])) AND ((“proximal kidney tubular dysfunction”[MeSH Terms] OR “kidney Tubular Dysfunction”[All Fields] OR “KTD”[MeSH Terms]), OR (Renal Tubular Dysfunction)) AND ((“polymorphism, single nucleotide”[MeSH Terms] OR (“polymorphism”[All Fields] AND “single”[All Fields] AND “nucleotide”[All Fields]) OR “single nucleotide polymorphism”[All Fields] OR (“single”[All Fields] AND “nucleotide”[All Fields] AND “polymorphism”[All Fields]))ORSNP[All Fields])) We selected manuals, publications (including abstracts) meeting the inclusion criteria. The systematic review was conducted in keeping with PRISMA guidelines (181).

2.2.2 Eligibility criteria

For inclusion in the review, studies must provide methods of mutational screening for SNPs, the target SNPs explored, rationale behind selecting those SNPs including if linkage disequilibrium was evaluated or not. Additionally, studies must clearly state a pre-specified outcome of ascertaining potential associations between TDF exposure, and risk for KTD as a factor of various SNPs of transport proteins involve in the bio-disposition of TDF. In this review, we included all studies regardless of their design, although all studies evaluated in this review (except one) had a case control design. Studies were excluded if they provide no detailed pharmacogenetic data, and failed to meet other inclusion criteria as outlined above.

2.3 Study quality evaluation

This was determined by a checklist and algorithm for methodological assessment of pharmacogenetic studies suggested by Jorgensen et al and this is discussed elsewhere (182).

2.4. Statistical analysis

In order to ascertain quality of studies under review, HWE was assessed in both cases and controls using the chi-squared tests. A P-value of <0.05 was considered statistically significant, and studies with deviation from HWE were defined as low-quality studies. Data pooling was performed with and without these studies to test the robustness of reported estimates. Heterogeneity of reviewed studies was ascertained by chi-squared tests. Overall, odd ratios for reviewed studies were determined using both fixed and random effect models. The Q-statistics considered in our analyses measures the heterogeneity across studies. Whilst I^2 quantifies the heterogeneity of these studies, and is expressed as a percentage of variability of effect sizes between studies. Where there was marked heterogeneity (as evidenced by high I^2), random effect models were considered. This ensures that the inevitable within, and between study variability were captured. We choose a random effects model because of heterogeneity of our data.

2.5.0 Results

2.5.1 Study selection

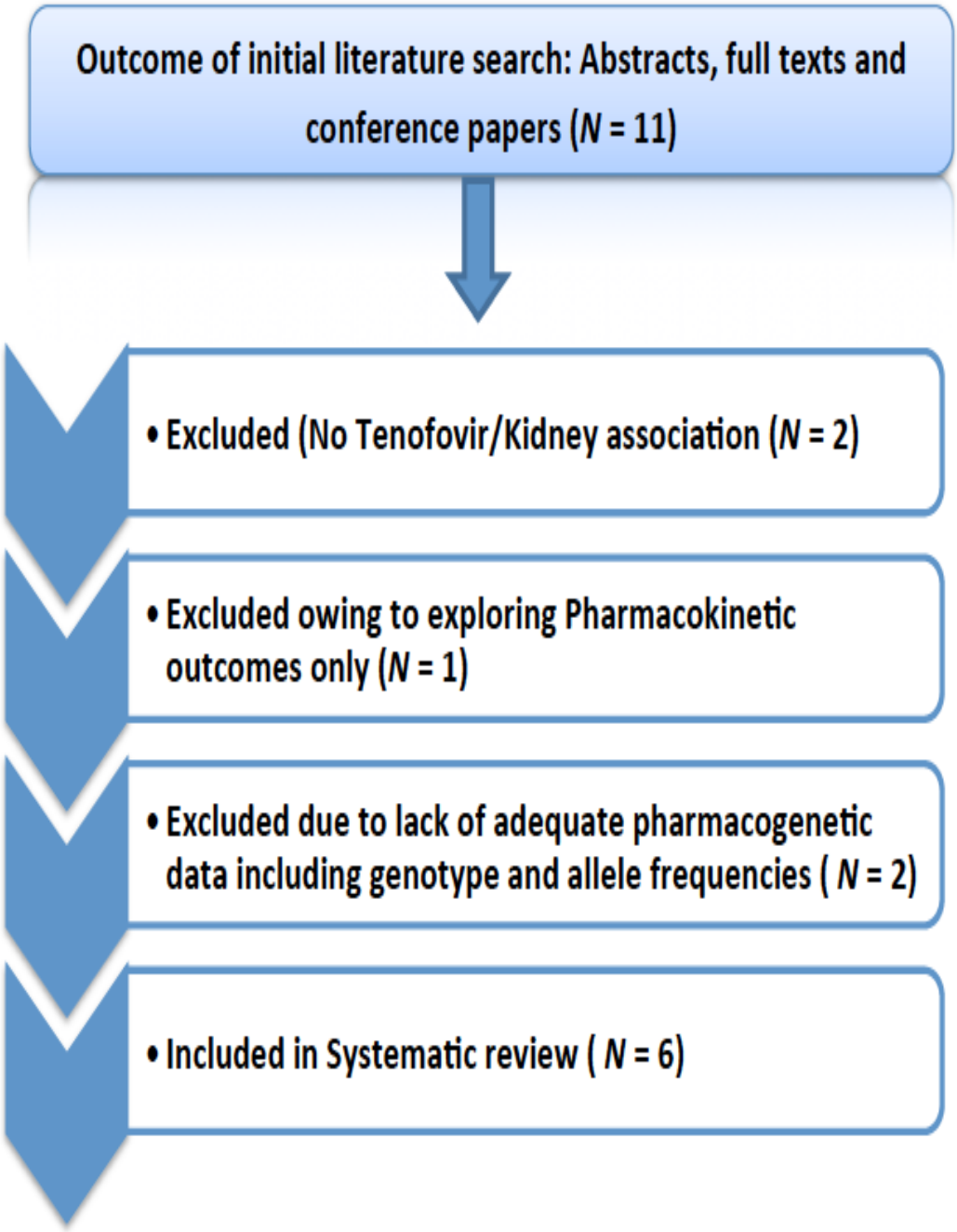
A comprehensive literature search retrieved eleven citations, out of which six met the inclusion criteria for analyses. There were no other previous systematic studies for comparison. We excluded the following referenced studies (183), and (184) because they were not designed to explore TDF-kidney toxicity association, and did not

report on this. da Rocha et al (185), and Manosuthi et al (186) reports provided incomplete allele and genotype frequency data, and were excluded from pooled analyses. The fifth report by Kiser JJ et al (187), explored TDF pharmacokinetic outcomes only and was excluded from pooled analyses. See figure 2.1 for flow diagram of reviewed studies.

2.5.2 Target single nucleotide polymorphisms

There were varied reasons expounded by investigators of the reviewed studies for evaluating specific SNPs. Izzedine et al (109) whose seminal work first established the probable role of genetics in TDF-related KTD, targeted various *ABBC2* (MRP2), and *ABCC4* (MRP4) SNPs based on the role they played in the pathology of other substrate transporters (109)

Figure 2.1: QUORUM Flow chart



In addition, their location within the basolateral membrane of the kidney tubules lent credence to their probable role in causation of TDF related KTD. In addition to this, other studies (107, 108, 110, 115) targeted the SNPs they evaluated based on functional significance, minor allele frequencies >5% in their respective populations. These includes reported protective effect of some haplotypes for example *ABCC2 4544G>A* (exon 32) which has been reported to be under-represented in patients with TDF-associated kidney toxicity (107). Additionally, *ABCC4 3463A>G,* and *ABCC4 4131T>G* were targeted because of their association with decreased renal TDF clearance (153) and high frequency in White populations (107) respectively. The *ABCC4 669C>T* SNP was studied owing to its high frequency of expression among patients with TDF-associated kidney toxicity (107). For this systematic review, we have focussed on the SNPs of the following transporter SNP's: *ABCC2* (MRP2), *ABCC4 (*MRP4), *ABCC10* (MRP7 rs2125739, and rs9349256), and *ABCB1 (*p-glycoprotein*).*

2.5.3 Study design and outcome definition

There was distinct concordance in the definition of KTD amongst four of the reviewed studies (107, 108, 110). They utilised a composite of serum and urinary parameters described extensively elsewhere (107) as surrogate markers of KTD. From the criteria utilised by the reviewed studies to estimate tubular injury, it is evident that the robust algorithm they employed for case adjudication and ascertainment supports their selection and consideration for this review. TDF induced KTD is currently a developing field and its current definition rely on utility of the composite criteria highlighted earlier (107). Five of the included reports utilised this, whilst the sixth report utilised the current validated point-of-care gold standard for the determination of kidney injury in HIV patients irrespective of ART

exposure (eGFR). The latter decision is based on current uniformity of eGFR <60mls/min/1.72m^2 as one of the internationally accepted surrogate markers of kidney dysfunction in both the general population as well as HIV positive patients on ART (its stated limitations not withstanding in these cohorts). We selected the six SNP's, and Table 2.1 gives a summary of various definition of KTD by the reviewed studies

2.5.4 Data Analysis

Data was abstracted by two independent reviewers (I, and Dr Sudeep Pushpakom of the Wolfson Centre for Personalised Medicine, the University of Liverpool) from studies included in the review, and entered into a Microsoft excel spreadsheet. Variables considered includes author, study design, sample size (including cases and controls), patients cohorts, effect size (OR), year of publication, target SNP, reported allelic or haplotypic associations, and Hardy Weinberg equilibrium (HWE). Effect size was determined from Pooled odd ratios (ORs) and confidence interval (CI) of selected studies. Dr Ben Francis (University of Liverpool) provided additional statistical input. Five of the reviewed studies had a case-control design with a uniform definition of cases and controls in four studies (107, 108, 110, 109) (utilising diagnostic criteria of Fanconi syndrome (107). Cases were defined as HIV positive patients on TDF who developed two of the following parameters (one of which must be a parameter for Fanconi syndrome) (107), with those not having any of these features classified as controls.

- Non-diabetic glucosuria (urine glucose level >300 mg daily)
- Total excretion of phosphorus (urine phosphorus × urine volume) >1200 mg daily

- Fractional tubular re-absorption of phosphorus (1 − [(urine phosphorus × plasma creatinine)/(plasma phosphorus × urine creatinine)]) <0.82
- Hyper-aminoaciduria (any amino acid in urine, with the exception of histidine, glycine and serine),
- β2-microglobulin (urinary β2-microglobulin > 1mg daily) [91]
- Fractional excretion of uric acid ([(urine uric acid × plasma creatinine)/ (urine creatinine × plasma uric acid)] × 100) >15%.

Fanconi syndrome encompasses glycosuria in non-diabetic patients, hyper aminoaciduria (except histidine, lysine and serine) and phosphaturia.

The fifth study (116) utilised thresholds of urinary median β-2 microglobulin (corrected for urinary creatinine excretion) in ascertainment of cases (KTD) and controls. β-2 microglobulinuria is an integral component of the diagnostic criterion for KTD/Fanconi syndrome discussed elsewhere in this thesis (107). Nishijima et al (115) utilised three thresholds of eGFR to classify kidney dysfunction into three distinct groups (>10ml/min/1.73m^2 fall in eGFR relative to baseline, >25% fall in eGFR, and eGFR <60ml/min/1.73m^2). We considered the third definition of kidney dysfunction i.e. eGFR <60mls/min/1.72m^2 in this review.

We estimated the population attributable risk of developing KTD following exposure to TDF (with possession of index SNP's) following pooling of data from the various studies that evaluated specific SNP's using the following formula:

PAR% = 100 × p (OR-1)/(p [OR-1]+1)

Where,

- P is the percentage attributable population risk
- p: Frequency of the index SNP allele in the control population cohort
- OR: odds ratio

Table 2.1: Definition of kidney Tubular Dysfunction (KTD) in studies included in the review

Study	Definition of kidney Tubular Dysfunction
Rodriquez-Novoa et al (2009) (107)	Presence of 2 abnormalities [1], one of which must be a Fanconi criteria (107)
Pushpakom et al (2011) (110)	Presence of 2 abnormalities [1], one of which must be a Fanconi criteria (107)
Izzedine et al (2006) (109)	Presence of 2 abnormalities[1], one of which must be a Fanconi criteria
Nishijima et al (2012) (108)	Presence of 2 abnormalities [1], one of which must be a Fanconi criteria (107)
Nishijima et al (2015) (115)	1. Decline in eGFR of >10 ml/min/1.73 m^2 relative to the baseline; 2. >25% decrement in eGFR relative to the baseline; 3. eGFR <60ml/min/1.73 m^2. (As determined by CKD-EPI equation)
Likanonsakul et al (116)	Median β-2 microglobulin (corrected for urinary creatinine excretion)

1.KTD abnormalities: non-diabetic glycosuria (urine glucose level, 1300 mg daily); total excretion of phosphorus (urine phosphorus x urine volume) >1200 mg daily; fractional tubular reabsorption of phosphorus (1 x [(urine phosphorus x plasma creatinine)/ (plasma phosphorus x urine creatinine)]) <0.82; hyperaminoaciduria (any amino acid in urine, with the exception of histidine, glycine, and serine), β2-microglobulinuria (β2-microglobulin level, >1 mg daily), and fractional excretion of uric acid ([(urine uric acid x plasma creatinine)/(urine creatinine x plasma uric acid)]x 100) >15%
2. Fanconi abnormalities: glycosuria in nondiabetic patients, hyperaminoaciduria, or hyperphosphaturia

2.5.5 Sample Size

The combined sample size of the study population was (N = 1426). This is made up of 250 cases and 1176 controls, with a median sample size of 152 (IQR 94, 380). None of the reviewed studies provided a pre-specified sample size/power calculation, potentially confounding the reported associations or lack of, between possession of these SNPs and risk for KTD following exposure to TDF. However, considering the minor allele frequency (MAF) of the SNP's evaluated in the studies, it is probable that they were underpowered to detect any significant associations.

2.5.6 Patient Populations/Stratification

Only one of the reviewed studies (115) provided stratification data beyond genotype model. Nishijima et al (115), additionally provided stratification based on dominant, recessive and additive models. None of the other studies reviewed in this review determined any potential confounding for cryptic populations. There were also a notably small number of black patients in all the six studies. This may have to do with centres where these studies were carried out (Europe Japan and Thailand). Two of the studies reported exclusively on Japanese patients (108, 115), and Thai patients only (116).

2.5.7 Reliability of Genotyping methods

There was uniformity in the genotyping assays employed across all studies. All reviewed studies utilised pharmacogenetic assays employing allelic discrimination using *TaqMan* 5-nuclease assays with standard protocols (107, 108, 110). There was however, no reference in the five reports (107, 108, 110) as to whether all study personnel were blinded to either the genotype or the outcome status. Similarly, despite the uniformity of genotyping methods, there were no comments regarding any attempts at determining genotyping quality control in any of the reviewed studies. These limitations represent an important source of potential confounding.

2.5.8 Hardy Weinberg equilibrium (HWE)

All studies performed statistical analyses for HWE of all evaluated SNP's (including *ABCC2, ABCC4, and ABCC10*) with no discernible deviation apparent or reported.

2.5.9 Mode of Inheritance

Apart from Nishijima et al (115), none of the other studies reported any determination of mode of inheritance of evaluated SNP's.

2.5.10 Adjustment for multiple comparisons

We did not carry out correction for multiple comparisons (Bonferoni) because of potential risk of type two errors given our small sample size.

2.6.0 Association between specific SNP variants and risk of Tenofovir induced Kidney tubular dysfunction in HIV positive patients

We systematically reviewed the influence of *ABCC2, ABCC4, ABCC10, and ABCB1* SNP's from various reports of HIV positive patients on TDF. Tables 2.2 to 2.4 give a summary of key determinants of variability of SNP's explored by various pharmacogenetic reports.

2.6.1 Influence of *ABCC2 24CC* (MRP) Polymorphisms on risk for KTD

A number of polymorphisms of the *ABBC2 (MRP2)* gene have been evaluated in recent published reports. Possession of the *ABCC2 24CC* genotype (MRP2, rs717620) was consistently associated with the risk for KTD in two of the evaluated studies (107, 108), Conversely, Nishijima et al (2015) (115) found no association between possession of this genotype and risk of TDF related KTD in these cohorts of patients. We found heterogeneity across all studies that explored *ABCC2 24CC* (MRP2, rs717620) SNP (I^2 = 51.2%, Q statistics = 8.19, P = 0.08). The pooled odds ratio (DerSimonian-Laird) for random effects model was 0.08, (95% CI = 0.05-0.13, P = <0.0001). This denotes association between possession of the *ABCC2 24CC* (rs717620), and risk for KTD in these cohorts of patients. It is noteworthy, that the Q statistics was lower than the key threshold of 16.9 (threshold for nine degrees of freedom). Table 2.3 gives the summary of fixed and random models of the studies in this report

Study, year (Country)	**Ethnicity**	**Design (numbers per group)**	**Sample size justification Provided**	**Definition of case group**	**Definition of comparison group**	**Exclusion criteria**	**Tenofovir exposure**	**Covariates**	**Genes investigated**	**SNPs investigated**	**HWE assessed at each locus**	**Statistical analysis**
Izzedine et al (109), 2006 (France)	Caucasian	Case control 13 cases, 17 controls	No	Cases and controls were defined as discussed in section 2.5.4	Cases and controls were defined as discussed in section 2.5.4	Not stated	One month	Duration of HIV, Age, and Male sex	*ABCC2* *ABCC4* *ABCB1*	*ABCC2 24CC* *ABCC2 1058* *ABCC2 1249* *ABCC2 3563* *ABCC2 3972* *ABCC2 4544* *ABCC4 559* *ABCC4 669* *ABCC4 912* *ABCC4 951* *ABCC4 969* *ABCC4 1497* *ABCC4 3310* *ABCC4 3348* *ABCC4 3609*	Yes	Logistics regression

Rodriquez-Novoa *et al (107)*, 2009 (UK)	Caucasian (90%)	Case control 19 cases, 96 controls	No	Cases and controls were defined as discussed in section 2.5.4	Cases and controls were defined as discussed in section 2.5.4	Not stated	Median of 34 months	Age, TDF exposure, Body weight, SNPs of genes encoding TDF transporters	*ABCC2* *ABCC4* *ABCB1* *SLC22A6* *SLC22A11*	*ABCC2 24CC* *ABCC2 1249* *ABCC2 3563* *ABCC2 3972* *ABCC2 4544* *ABCC4 669* *ABCC4 3463* *ABCC4 4131* *ABCB1 3435* *ABCB1 1236* *SLC22A6* *SLC22A11*	Yes	Logistics regression
Pushpakom *et al (110)*, 2011 (UK)	Caucasian 90%	Case control (19 Cases, 96 controls)	No	Cases and controls were defined as discussed in section 2.5.4	Cases and controls were defined as discussed in section 2.5.4	Not stated	Median of 34 months	Age, TDF exposure, Body weight, SNPs of genes encoding TDF transporters	*ABCC10*	*ABCC10 (rs2125739)* *ABCC10 (rs9349256)*	Yes	Logistics regression

Nishijima *et al (108)*, 2012 (Japan)	Japanese (100%)	Case-control (19 cases, 171 controls)	No	Cases and controls were defined as discussed in section 2.5.4	Cases and controls were defined as discussed in section 2.5.4	(1) Active infection, (2) malignancy, (3) diabetes (4) alanine aminotransferase 2.5 times more than the upper limit of normal. (5) Estimated glomerular filtration rate (eGFR) <50 mL/minutes. (6) Patients without consent to the study	71.5 weeks	Sex, Age, Gender, body weight, duration of TDF exposure, SNPs of genes encoding TDF transporters	*ABCC2* *ABCC4* *ABCC10* *ABCB1* *SLC22A6*	*ABCC2 24CC* *ABCC2 1249* *ABCC2 2366* *ABCC2 2934* *ABCC4 559* *ABCC4 912* *ABCC4 2269* *ABCC4 3348* *ABCC4 4135* *ABCC4 4976* *ABCC10 rs2125739* *ABCC10 rs9349256* *ABCB1 2677 T>A* *SLC22A6 rs11568630*	Yes	Logistics regression

Danjuma *et al*, in press (UK)	Caucasian (73.7%)	Case-control (15 case and 43 controls)	No	Cases were defined by urinary RBPCR >17	Controls were defined as patients with RBPCR <17	Not stated	583 days	Age, weight, duration of TDF exposure, SNPs of genes encoding TDF transporters	*ABCC2* *ABCC4* *ABCC10* *SLC22A6* *SCL22A11*	*ABCC2_24CC* *ABCC4 669* *ABCC4 3463* *ABCC10 (rs2125739)* *ABCC10 (rs9349256)* *SLC22A6* *SLC22A11*	Yes	Logistics regression
Nishijima et al (115) 2015 (Japan)	Japanese (100%)	Prospective cohort	No	Cases were defined as patients with eGFR >10ml/min/1.73m2 decrement in eGFR relative to baseline, >25% decrement in eGFR, and eGFR <60ml/min/1.73m2	Controls were patients with eGFR greater the thresholds defined earlier	Exclusion criteria provided	3.66 years	Age, weight, BMI, HIV VL, CD4 count, duration of TDF exposure, SNPs of genes encoding TDF transporters	*ABCC2* *ABCB1*	*24C>T* *1249G>A* *2677T>A*	Yes	Logistic regression

Likanonsakul et al (116) Thailand (2016)	Thai (100%)	Case control; *n* = 273 (cases = 54, controls = 219)	No	Cases and controls were defined based on urinary β-microglobulin thresholds	Cases and controls were defined based on urinary β-microglobulin thresholds		At least 12 months	Age, weight, duration of TDF exposure, and genes encoding TDF transporters	*ABCC2* *ABCC4*	*-24CT* *1249* *T4976C*	Yes	Logistic regression

RBPCR: urinary retinol binding protein creatinine ratio (corrected for urinary creatinine excretion

Table 2.2: Summary of demographic and pharmacogenetic characteristics of evaluated studies

As reported by previous published mechanistic/pharmacogenetic studies (107, 108, 109), MRP2 (*ABCC2)* is not a transporter for TDF. Rather, putative transporters for TDF include MRP4 (*ABCC4),* and MRP7 *(ABCC10)* (110). Probable suggestions for this mechanistic dissonance have already been dealt with in section 1.4.1 of this thesis. Regardless of pharmacogenetic mechanism for this paradox, the consistency of association across these reports suggests a potential predictive role for the *ABBC2 24CC* (rs717620) genotype. Other evaluated SNPs of *ABCC2* (MRP2) include *ABCC2 1249GA* (rs2273697) (107), (108),(109), *ABCC2 3563TA* (rs8187694) (107), (109). It is noteworthy, that no consistent association has been reported with these SNPs. Figure 2.2 depicts a forest plot of studies evaluating the *ABCC2 24CC* (rs717620) SNP.

Table 2.3: A random and fixed effects model summary of evaluated studies

Pharmacogenetic and statistical consideration					**Random Effects Model**			**Fixed Effects Model**		
SNP ID	**Target Genotype /Allele**	**Genotype/Allele Frequency [1]**	**I^2 (%)**	**Q-statistics**	**Effect Size**	**Confidence interval (95%)**	**P**	**Effect Size**	**Confidence interval (95%)**	**P**
ABBC2 24CC (MRP2 rs717620)	CC	135	51.2	8.19	0.08	0.05-0.13	<0.0001	0.08	0.06-0.11	<0.0001
ABCC10 (MRP7, rs9349256)	GG	56	0	0.93	0.04	0.03-0.06	<0.0001	0.04	0.02-0.06	<0.0001
ABCC10 (MRP7, rs2125739)	CC	27	0	0.93	0.12	0.07-0.20	<0.0001	0.12	0.07-0.19	<0.0001

1. Combined target genotype/allele frequencies of the reviewed studies

I^2 = measure that aims to quantify heterogeneity of the studies. The higher the value, the more heterogeneous the studies are.

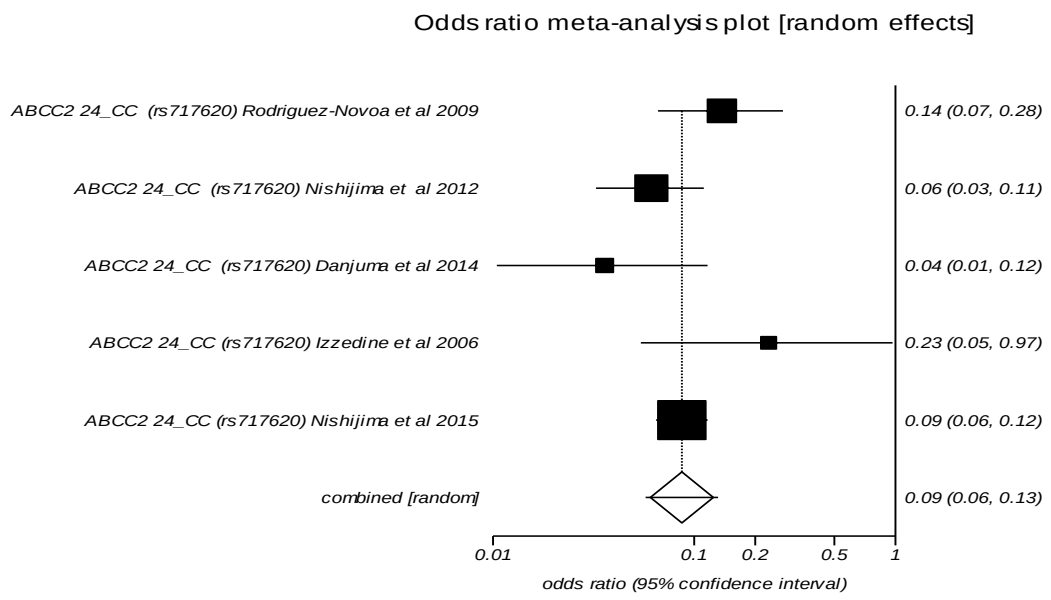


Figure 2.2: Forest plot of studies exploring association between *ABCC2 24CC* single nucleotide polymorphism and risk of TDF induced kidney tubular dysfunction in HIV positive patients

2.6.2 Role of *ABCC10* (MRP7) gene polymorphisms in Tenofovir induced Kidney tubular dysfunction

Three of the reviewed studies in this report explored the potential association between possession of *ABCC10* SNPs (rs9349256 and rs2125739) and risk for KTD following TDF exposure in HIV positive patients (108), (110), and (115). There was no significant heterogeneity in the evaluated *ABCC10* SNPs (MRP7, rs9349256 and rs2125739) in regards to their effect sizes (I^2 = 0%, Q statistics of 0.93, P = <0.62 vs. I^2 = 0%, Q statistics = 0.93, p = <0.65 respectively). There was significant association between possession of the intronic G allele of the *ABCC10* (rs9349256), and C allele of the *ABCC10* (rs2125739) and risk for KTD (Mantel-Haenszel pooled odds ratios for the fixed effects models = 0.04, CI = 0.02-0.6, p = <0.0.0001; 0.12,

and CI = 0.07-0.19, p = <0.0001 respectively) figures 2.3 and 2.4. Table 2.3 gives the estimate of the predictive models including the random effects model. In a recent combined mechanistic/pharmacogenetic seminal report, Pushpakom et al (110) first established the association between possession of *ABCC10* genotypes and combined *ABCC2-ABCC10* haplotypes with the risk of TDF induced KTD. There was an over-representation of the intronic *G* allele (rs9349256) in the KTD cohort (OR 2.3; CI, 1.1–5.3; P = 0.02), compared with non-KTD controls. Conversely, the exonic non-synchronous SNP (*ABCC10* rs2125739) was found to be only marginally associated with risk for KTD (OR, 2.0, P = 0.05).

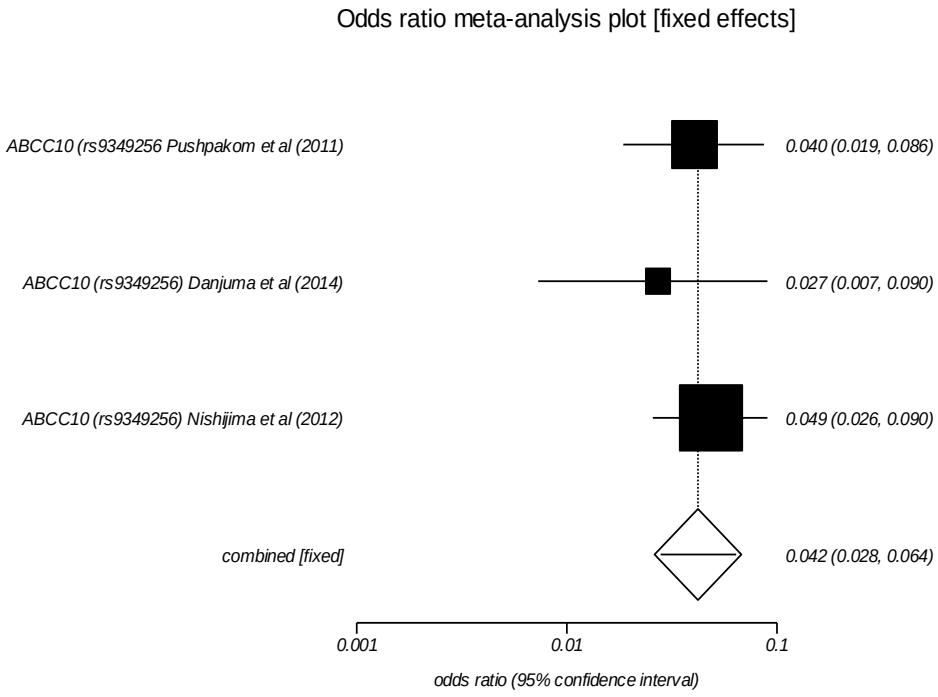


Figure 2.3: Forest plot of studies evaluating single nucleotide polymorphisms of *ABCC10* (MRP7, rs9349256) gene and risk of Tenofovir induced kidney tubular dysfunction in HIV positive patients

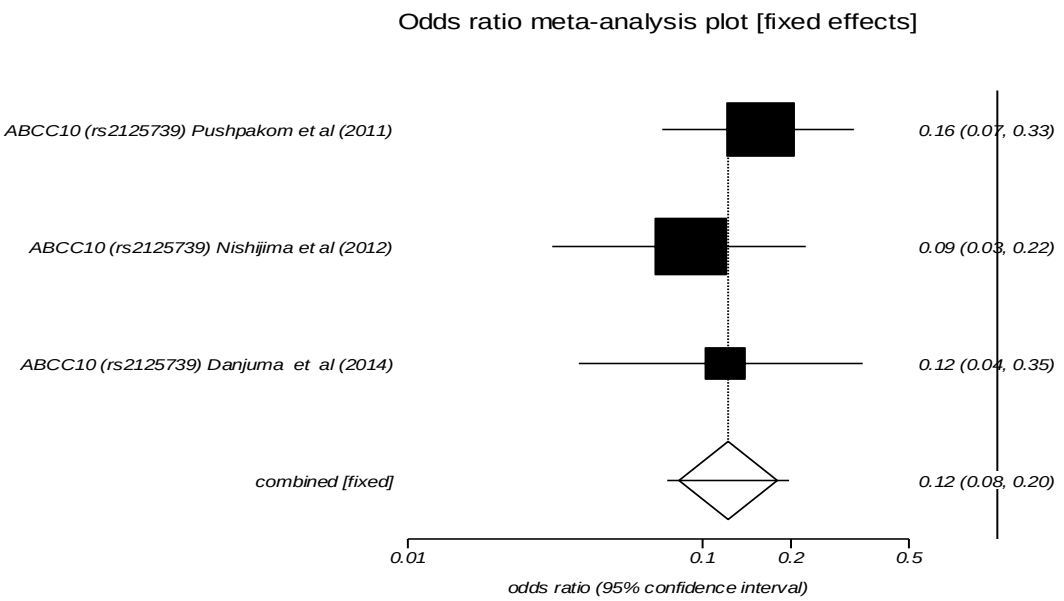


Figure 2.4: Forest plot of studies evaluating single nucleotide polymorphisms of *ABCC10* (MRP7, rs2125739) gene and risk of Tenofovir induced kidney tubular dysfunction in HIV positive patient

2.7.0 Discussion

There has been considerable interest regarding the association of TDF with development of KTD in HIV positive patients. Whilst the exact clinical and laboratory phenotypes have continued to be subjects of intense debate, possession of certain single nucleotide polymorphisms of genes encoding proteins involved in TDF transport seems to confer increased risk for KTD (107, 108, 110). In separate reports, Rodriquez-Novoa (107) et al, and Pushpakom et al (110) first reported the association between possession of the *ABCC2 24CC* (MRP2, rs717620) and *ABCC10* (MRP7, rs2125739, rs9349256) SNPs respectively with increased risk for KTD in these cohorts of patients. Subsequent reports have since suggested varying degrees of association between the *ABCC2* 24CC, the two *ABCC10* SNPs (rs2125739 and rs9349256), and KTD risk in these cohorts of patients.

Table 2.4: *ABCC2* and *ABCC10* SNP frequencies and distribution amongst KTD cohorts in HIV positive patients exposed to TDF

Studies	**Design**	**Population**	***N***	**Gender (male) %**	***ABCC2* Genotype Frequencies**			***ABCC10* Genotype frequencies**		
								rs9349256	rs2125739	
								GG GA AA	CC CT	TT
Rodriguez-Novoa et al (2009) (107)	Case control	HIV positive patients (Hospital Cohort)	115	78.9	16	2	1	- - -	- -	-
Nishijima et al (2012) (108)	Case control	HIV positive patients (Hospital Cohort)	190	94.7	18	1	0	4 9 6	15 6	0
Pushpakom et al (2011) (110)	Case control	HIV positive patients (Hospital Cohort)	115	98	16	2	1	-	22	- -
Nishijima et al (2015) (115)	Prospective cohort §	HIV positive patients (Hospital cohort)	703	95.6	83	38	5	--	--	--
Danjuma et al (in press)	Case control	HIV positive patients (Hospital Cohort)	81	43	24		7	13 17	7	15
Izzedine et al (2006) (109)	Case control	HIV positive patients (Hospital Cohort)	30	92	9	3	1	- -	--	--

§. We considered the third out of the three outcomes used to define kidney injury in this study (i.e. eGFR<60mls/min/1.72m^2)

It is noteworthy however, that in recent pharmacogenetic analyses of a large prospective cohort of HIV positive patients on TDF based regimen Nishijima et al (115) reported no significant association between possession of the *ABCC2 24CC* genotype and risk for KTD. How the relatively large sample size of this study and its mode of adjudication of kidney dysfunction (eGFR rather than novel markers of tubular dysfunction) affect this outcome remains uncertain. Additionally, the percentage variability (attributable to possession of these SNP's) in risk of developing KTD following TDF exposure in light of other potential confounders (including concomitant medications, and HIV disease process itself) remains unsettled. Utilising pooled estimates from constituent studies, we found an attributable risk of about 9.9% of developing KTD with possession of the *ABCC2 24CC* genotype following exposure to TDF in HIV positive patients. Overall, there appears to be over representation of the male population amongst carriers of the *ABCC2 24CC* genotype (107). About 78-98% of the KTD cohorts for example in the Six published studies evaluating this SNP were males (107, 108, 115). Whether this male over representation has to do with previously suggested mitochondropathy as a mechanism for TDF induced kidney injury is open to debate. Mitochondrial DNA is highly mutagenic, and its inheritance is sex-linked (exclusively from the mother). How SNPs of these impacts on TDF suggested mitochondropathy should perhaps be considered as additional themes in future pharmacogenetic work in this area. It is noteworthy that none of the reviewed studies other than Nishijima et al (2015) (115) in this systematic review reported on correction/stratification for cryptic populations. However, despite stratification, none of the four genetic models showed any significant association between possession of the *ABCC2 24 CC* SNP, and risk of TDF induced KTD (115). Most of the studies were carried out in predominantly

white populations with two of the studies reporting on exclusively Japanese and Thai patients respectively. How much of the variability in susceptibility of this patient population to TDF induced kidney dysfunction is explained by possession of these SNPs of transport proteins remains unknown. The consistency of these reported associations would suggest an underlying effect, the magnitude of which is currently difficult to ascertain. Issues with sample size, and methodological qualities of these reports may have either blunt or accentuate these effects/associations. In a recent report, Jorgenson et al (182) have attempted to address the obvious flaws of these reports in common with most recent pharmacogenetic reports. Some of the recommendations from this report includes, a checklist of key methodological and genotypic issues that needs to be addressed by would-be trialists in the design of future pharmacogenetic studies exploring TDF exposure with risk for KTD. Similarly, uncertainty would continue to remain until an all-encompassing, readily available, and accessible point-of-care outcome mode of adjudication of KTD is agreed upon.

2.7.1 Limitations

Our analysis is limited by the relatively small sample size of the studies systematically reviewed. This perhaps reflects the fact that this is an emerging field of ART pharmacogenetics. Additionally, the absence of emphasis on the genotyping quality and non-blinding of study personnel as to the various genotypes and study outcomes undoubtedly represent a significant source of confounding. Furthermore, all the reviewed studies considered a candidate gene-based approach to establishing association between TDF exposure and risk of kidney injury in these cohorts of patients. What has not been explored thus far is the strength and robustness of these associations utilising a genome wide association study (GWAS). From studies in

both the general population as well as HIV positive cohorts, GWAS has been shown and does have the potential to identify other putative genetic markers that may have been missed by hypothesis-based candidate gene approach due to SNP selection bias. Furthermore, the lack of a standardised, uniform, acceptable definition of cases and controls may have under or overestimated the strength of reported associations

2.7.2 Conclusion

We have shown from this systematic review that despite limitations highlighted earlier, possession of *ABCC2 24CC* (rs717620), *ABCC10* (rs2125739, and rs9349256) SNPs were associated with risk for KTD in HIV positive patients exposed to TDF.

CHAPTER 3

THE DETERMINATION OF THE PATTERN OF TENOFOVIR INDUCED-KIDNEY INJURY IN HIV POSITIVE PATIENTS: AN EXAMINATION OF THE MEDICINES AND HEALTHCARE PRODUCTS REGULATORY AGENCY (MHRA) OBSERVATIONAL COHORT

3.0 Introduction

I set out to conduct a descriptive analyses of yellow card records of HIV positive patients on TDF based regimen who developed adverse events, and had them reported to medicines and healthcare products regulatory agency (MHRA).

3.1.1 Methods

3.1.2 Study design

This is a descriptive analysis of yellow card records submitted to the MHRA. The Yellow Card scheme is the pharmaco-vigilance tool employed by the MHRA to encourage spontaneous and voluntary reporting of all suspected adverse drug reactions by healthcare providers, the pharmaceutical industry, and patients (since 2005) in the United Kingdom (188). This data is subsequently classified into an online repository called the adverse drug reaction on-line information tracking (ADROIT) database and updated on a regular basis as additional reports are submitted (188). Periodic evaluation of this data is carried out to identify any adverse events signals or potential hazards associated with medicines currently in clinical use. Where adverse signals are ascertained or suspected, they are communicated to both healthcare providers and patients to prevent any further harm and protect public health (188).

For this study, category II Yellow Card reports of HIV-positive persons taking TDF as part of their ART regimen and spontaneously reported to the MHRA and Commission on Human Medicines (CHM) between 2001 and 2010 was obtained and analysed. Category II data refers to data with potential patient/reporter identifiers. They include patient's medical history, dates of drug administration, reported reaction as well as pre and post exposure blood test results. Utilisation of these data therefore require prior authorisation. An application to obtain and analyse these data

was submitted to independent scientific advisory committee (ISAC) of the MHRA. Following exhaustive review, approval was granted to obtain and analyse these data. Data was received in form of Microsoft excel spread sheets with key variables such as adverse drug reaction number (ADR), duration on TDF, route of administration, description of the drugs adverse event, action taken by the reporting team, ADR outcome, weight, age, unclassified laboratory data (including serum creatinine, urea, serum phosphate, serum calcium, HIV viral load, and CD4 count). Urinary parameters accompanying the data include urinary amino acids (where available), urine protein creatinine ratio (PCR), results of urine dipstick including glycosuria and proteinuria. Not all patients had complete laboratory dataset before commencing, whilst taking, and after TDF exposure. Before classification, the data was cleaned to remove duplicate entries. Cases that were adjudicated by original reporting source to be "Fanconi syndrome", "acute kidney injury" (reported as "increased creatinine" "reduced GFR", "acute renal failure") or chronic kidney disease/injury were analysed. In addition reports with outcomes such as "nephrogenic diabetes insipidus", proteinuria, glycosuria and amino aciduria (with or without other components of Fanconi syndrome) were classified as suspected kidney tubular dysfunction (KTD) and analysed as a sub-group. Where numerical eGFR was reported as the marker of kidney dysfunction, I further standardised this by estimation with the CKD-EPI equation and presented as mls/min/1.73m^2.

3.1.3 Definition of Tenofovir associated kidney Injury

I utilised the definition of kidney tubular dysfunction (KTD) and Fanconi syndrome as variously discussed elsewhere in literature (107).

Additionally, CKD was defined as estimated glomerular filtration rate (eGFR)/creatinine clearance (estimated by Cockcroft-Gault equation) <60 mls/min/1.73m^2 over a 3-month period.

Cases where alternative causes for kidney impairment were reported (including nephrotoxic drugs and intrinsic kidney disease amongst others) were excluded from the analyses.

Resolution of kidney dysfunction was defined as eGFR/creatinine clearance >60 mls/min/1.73m^2 from baseline, or normalisation of makers of KTD/Fanconi syndrome (highlighted above) on follow up assay.

3.2.0 Patient recruitment and screening

3.2.1 Inclusion criteria

- HIV positive
- On TDF based regimen
- Any stage of kidney disease

3.2.2 Exclusion criteria

Yellow card reports without evidence of TDF exposure

3.2.3 Statistical Methods and Data handling

Demographic, laboratory, and clinical characteristic were compared using Student T- and Chi-squared tests for parametric and non-parametric variables as appropriate. Descriptive results of variables were presented as Median ± interquartile range, Mean ±standard deviation as appropriate. All statistical analyses were carried out with SPSS software 22 (version Inc. Chicago, Illinois, USA).

3.3.0 Data management

As discussed in earlier section of this chapter, data were received unclassified from MHRA in form of a Microsoft excel spreadsheet. This was classified as earlier described and stored in the secured computers of department of clinical pharmacology, the University of Liverpool. Access to these records are logged and restricted to the principal and co-investigators only.

3.4 Ethics approval

An initial application for authorization to utilize ADROIT data in order to carry out this analysis was submitted to the MHRA. This was reviewed by ISAC and a provisional approval granted subject to compliance with suggested amendment to the study protocol. Following implementation of suggested amendments, a further review by ISAC resulted in full authorization to proceed with data acquisition from information technology unit of MHRA.

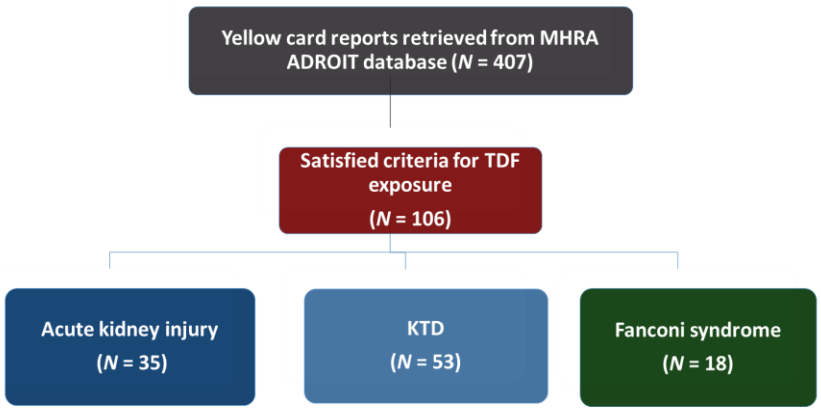


Figure 3.1: Classification of MHRA data into various kidney phenotypes

3.5.0 Results

The demographic and clinical characteristics of the study population are given in Table 3.1. Over a 9-year period (2001-2010), a total of 407 category II Yellow Card reports of patients with suspected kidney related ADRs due to TDF were reviewed.

One hundred and six (106) satisfied the definition of acute kidney injury (33%), Fanconi syndrome (17%), and kidney tubular dysfunction (50%), figure 3.1. There was significant correlation between eGFR (estimated by the EPI-CKD equation) and age in this patient cohort (r = -0.41, *P* = 0.01).

Out of the 106 reports analysed, 33 (31.4%) patients required hospitalisation due to TDF-related kidney adverse events, with a mortality of 18.2% (6 out of 33 patients), figure 3.2. There was a statistically significant difference (P = 0.01) in the rates of hospitalisation between the 3 TDF related kidney phenotypes. Patients classified as Fanconi syndrome had the highest frequency of hospitalization (55.6%). Fanconi syndrome represents the extreme phenotype of TDF related kidney toxicity.

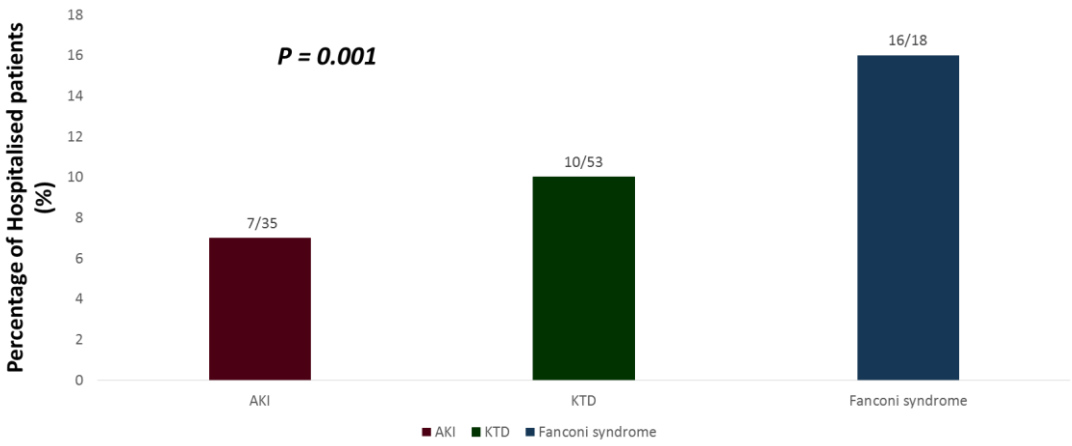


Figure 3.2: Morbidity of various TDF Kidney phenotypes amongst MHRA patient cohorts

3.5.1 Amino Aciduria and TDF exposure

About 2.8% of the studied cohort had urinary amino acid abnormalities. However, the nature of amino acids excreted in urine in these patients was not reported. In all cases, cessation of TDF was associated with normalization of adverse kidney parameters (including amino aciduria).

3.5.2 Proteinuria and TDF exposure

Two patients had significant Nephrotic range proteinuria out of the 106 patient cohorts examined. The first patient had 4.5gram/24hours proteinuria thirteen months after commencing TDF. There were no records of normalisation of kidney function, and or persistent proteinuria following cessation of TDF in this patient. Whilst the second patient had 2.59 grams proteinuria in 24 hours, with kidney function (including 24 hour urinary protein excretion) returning back to normal following cessation of TDF. In both cases, the diagnostic threshold for significant proteinuria was attained.

Table 3.1: Demographic and clinical characteristics of the study population (N = 106)

Characteristics	**All patients**	**AKI[a] (*N*=35)**	**KTD [b] *(N=53)***	**Fanconi Syndrome (*N* =18)**	***P***
Age (years) *Mean (±SD [c])*	41 (±13)	44.1 (±14.1)	39.1 (±12.1)	45 (±11.7)	0.16
Gender (Male) *(N)*	77	27	38	12	0.6
Ethnicity (White) *N*	4	-	*2*	2	0.41
Hepatitis BsAg (Positive) *N*	2	-	*2*	-	--
CD4 cell count (cells/mm^3) *Median (IQR[D])*	192 (117, 259)	200 (55, 241)	200 (117, 259)	232 (113, 305)	0.86
HIV RNA viral load (copies/ml) *Median (IQR)*		1165 (244, 2710)	4676 (63.8, 29355)	811 (811, 811)	0.90
Duration on TDF (Days) *Median (IQR)*	316 (120,740)	374.5 (51.5, 813.7)	277 (122.5, 521.5)	419 (246, 1000)	0.46
Protease Inhibitors (PI) exposure *N (%)*	4 (3.3)	0	3	1	0.36
Weight (Kilograms) *Median (IQR)*	61 (50, 73)	63 (51.5, 76)	60 (50, 70)	55 (48, 74.3)	0.59
eGFR (ml/min/1.73m^2) *Median (IQR)*	34 (12.2, 58.2)	29 (25, 75)	44 (12, 64)	41 (18, 61)	0.58
Serum Creatinine (µmol/L) *(Median, IQR)*	183 (127, 371)	221 (146, 379)	133 (123, 500)	145 (110, 523)	0.48
Urine PCR[e] (mg/mmol) *Median (IQR)*	62 (4.4, 113.5)	62 (62, 62)	48.9 (4.1, 114.4)	--	1.0
Alkaline phosphatase (IU/L) *(Median, IQR)*	265 (196, 330)	286 (187, 329)	324 (228, 324)	204 (164, 204)	0.29

Data presented are median (interquartile range) for quantitative variables and number of patients (percentage) for qualitative variables as appropriate. *a: acute kidney injury; b: Based on RBPCR > or < 17* µg/mmol*; c: Standard deviation; d: Inter-quartile range; e: Urine protein creatinine ratio*

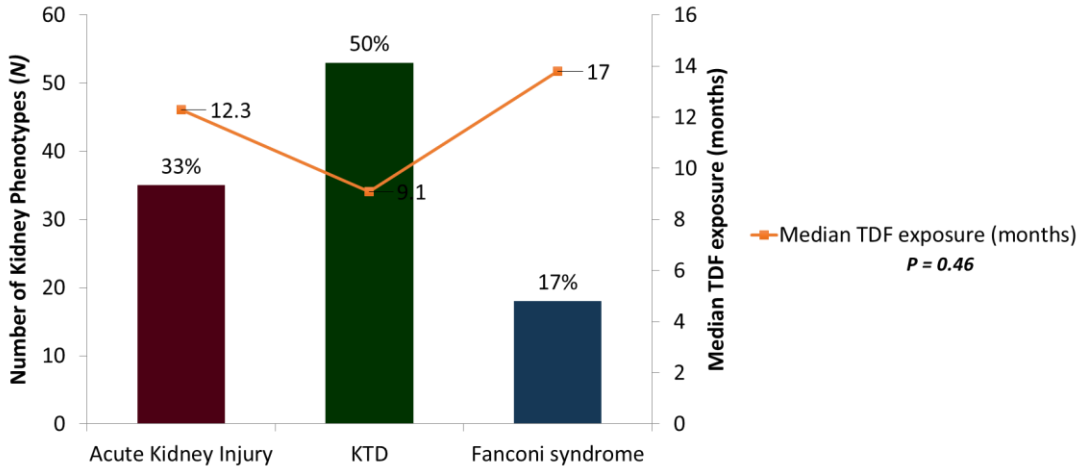


Figure 3.3. TDF exposure (in months) amongst various Kidney phenotypes in the MHRA cohort

There were limited data available for HIV protease inhibitor exposure (3.5 %). Only four patients of the entire series examined had data on PI exposure. Of these, patients on boosted Atazanavir (n = 2) had KTD, whilst those on boosted Lopinavir and Indinavir had Fanconi syndrome and KTD respectively. The median time between TDF exposure and onset of kidney related adverse effect was 316 days (IQR, 120,740). See figure 3.3

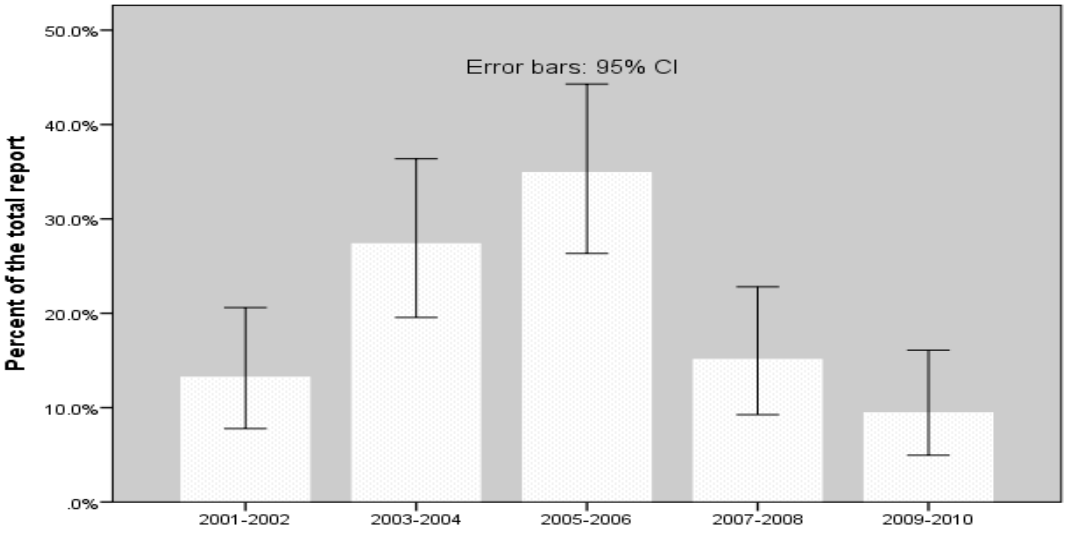


Figure 3.4: Year of report of Kidney related adverse effects to the MHRA in HIV-infected patients exposed to Tenofovir (2001-2010)

Nearly half (48.1%) of a total of 106 Yellow Card reports of TDF related kidney adverse effects analysed reported resolution following withdrawal of the drug. None of the patients fulfilled criteria for CKD before exposure to TDF but four patients (6.2%) out of the total number of reports had eGFR <60mls/min/1.73 m^2 consistent with CKD even after withdrawal of TDF. The report of TDF kidney related adverse effects from various community and clinical sources to the MHRA peaked between 2005-2006 as shown in figure 3.4.

3.6.0 Discussion

This represents the first attempt at interrogation of MHRA records to ascertain the pattern of kidney injury in HIV-positive persons exposed to TDF. Despite the likely under reporting of cases, this study provides additional insight into the pattern of kidney involvement amongst HIV positive persons exposed to TDF, and supports this association reported from observational and randomised clinical trials. TDF has well-established efficacy and safety profile for the treatment of HIV patients, but a propensity to cause various kidney syndromes have continued to be a subject of great

concern as expounded in other areas of this thesis (144, 145). The high number of TDF related ADRs evident between 2005-2006 in this study (as shown in figure 3.3), and subsequent fall thereafter would suggest periods of increasing awareness, increased monitoring, and resultant caution with the use of TDF. Alternatively, it may reflect the enthusiasm sometimes seen with early experience with a new drug, and the associated tendency to report on any new untoward adverse effects (AE's). However, as often is the case, with more clinical experience with it, there is a waning tendency to report less on these AE's. I was unable to ascertain the impact of protease inhibitor (PI) exposure on the pattern of TDF-related injury in this cohort owing to missing data on this variable. Overall, only 4 patients of the entire series had data on PI exposure. Nevertheless, patients who were on boosted Atazanavir (*n* = 2) had KTD, whilst those on boosted Lopinavir and Indinavir had Fanconi syndrome and KTD respectively. The role of PI exposure in the pathogenesis of TDF-related injury has continued to be a subject of intense mechanistic and therapeutic debate (154, 153, 171). In a recent report exploring the Swiss HIV study cohort database, contemporaneous administration of TDF with boosted Atazanavir or Lopinavir was associated with a greater initial decline in e-GFR compared to TDF/EFV regimen (189). Similarly Cao et al (190), recently reported a greater rate of decline in renal function in Chinese patients on TDF+PI/r based ART regimen compared with their cohorts on non-TDF regimen at 48 weeks. Amongst suggested mechanisms for the role of PIs in the TDF-related kidney injury includes PI-mediated increase in plasma concentration of TDF (191). In contrast to earlier reports, this study showed no preponderance of the KTD/Fanconi phenotype in male sex. The fact that only about 50% of patients in this study were reported to have regained normal kidney function is very interesting. Four patients out of the examined cohort had CKD. The

underlying primary/secondary kidney disease in the patients with CKD in the reports was unstated. The mean duration of restoration of kidney function following cessation of TDF is variable (192). All of the reported mortality in this study were due other HIV related complications and not attributable to progressive kidney disease or TDF exposure. Similarly though 6 out of the 33 patients hospitalized for TDF related adverse effects died in this report, it still remains uncertain if these were mainly as a result of TDF kidney adverse effects only, or if concomitant burden of associated comorbidities largely drove this. Two patients had Nephrotic range proteinuria, there were no further data or information as to the definitive cause of this. TDF has been associated with varying degrees of proteinuria, but association with frank Nephrotic syndrome is still yet to be established. One patient died of intractable metabolic acidosis with no additional details to support the role of TDF exposure or other alternative diagnoses. Another patient had associated limb weakness with muscle biopsy demonstrating COX negative fibres suggestive of drug-induced mitochondrial toxicity. The third patient had acute pancreatitis with severe metabolic acidosis.

3.6.1 Limitations

The limitation of this study reflects problems encountered by previous attempts at exploring such data schemes. These include its descriptive design, difficulties with accurately estimating the prevalence of TDF related kidney injury, certainty in establishing causality between TDF exposure and kidney injury, and incomplete records of reported events amongst others. The significance of under reporting of yellow cards as a limitation of this study needs to be emphasized, as it remains an important confounder to the outcome of this study. Not all professionals statutorily required to send yellow card reports of patients on TDF who develop ADR were well

informed about the need for this. And even where there was an understanding of need, often the report sent was not exhaustive and fails to support the clinical circumstances underlying the ADR. Additionally, very often with early experience of a new drug, there is a tendency for increased yellow card reports, which then wanes as more clinical experience with the drug becomes established. This may have explained the varying time-related reporting pattern with TDF in our report. In addition the absence of a universally accepted validated definition of what constitutes tubular dysfunction in HIV-positive persons on TDF makes objective adjudication of the Yellow Card reports difficult.

3.6.2 Conclusion and future perspectives

The pattern of TDF associated kidney adverse effects observed in this population series mirrors that reported in both hospital cohorts, and randomized clinical trials. Cessation of TDF was associated with complete restoration of kidney function in up half of the patient population in this report.

CHAPTER 4

ASSOCIATION BETWEEN *ABCC2* and *ABCC10* SUB-FAMILY GENETIC POLYMORPHISMS, AND RISK OF KIDNEY INJURY IN HIV POSITIVE PATIENTS EXPOSED TO TENOFOVIR DISOPROXIL FUMARATE (TDF): A CANDIDATE GENE STUDY

4.0 Background

4.1 Tenofovir transport

TDF is excreted by the kidneys through a combination of glomerular filtration, and active tubular secretion into the kidney tubular lumen. Two groups of drug transporters have been identified to facilitate TDF kinetics. Influx and efflux transporters such as organic anion transporters (OAT), and multi drug resistance proteins (MRP) transports TDF in and out of kidney tubular cells respectively (150, 155), figure 4.1.

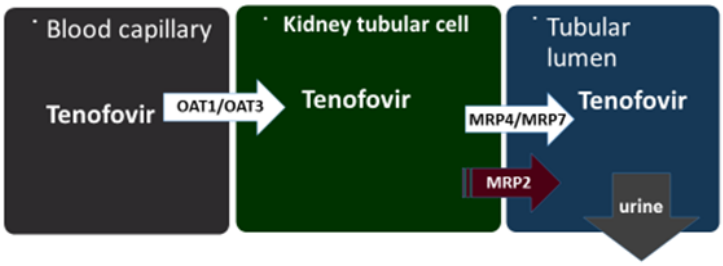


Figure 4.1: Schematic representation of TDF transport proteins (MRP2 is represented in red as it has not been established as a TDF transporter despite genetic association SNP's of gene encoding it)

4.1.1 Organic Anion Transporters (OAT)

Organic anion transport (OAT) (150, 155) proteins are expressed at the basolateral aspect of kidney tubular cells (KTC) and have been established from a number of mechanistic studies to transport TDF into these cells from blood capillaries. Multi-drug resistant (MRP) proteins mediate the transport from KTC's into the tubular lumen(155). Influx transporters OAT1 and OAT3 (encoded by the *SLC22A6* and

SLC22A8 genes respectively) have thus far been shown to be the key transporters involved in TDF transport into KTC's (193). To date no single nucleotide polymorphisms (SNPs) of their genes have been shown to either impact on TDF transport, therapeutics, or toxicity.

4.1.2 Multi-drug resistant protein (MRP) transport systems

This class of drug transporters is comprised of about seven members, and are involved in organic anionic transport of a varied number of drugs and other xenobiotics across intracellular and extracellular membranes (194). They are principally found in the liver, kidneys, and intestinal wall (194). Widely distributed in various ethnic populations as depicted in table 4.1, their production is encoded by a superfamily of genes called antigen-binding cassettes (ABC) (194). These are designated subfamily C, member 2 for *ABBC2*, member 4 (*ABCC4*), and member 10 (*ABCC10*) etc. They have been shown to transport a number of ART drugs, notably TDF (MRP4/*ABCC4* (143), MRP7/*ABCC10* (110). Mutations in these genes have been reported from a number of pharmacogenetic studies to impact on the transport of ART drugs as expounded in other sections of this thesis. Figures 4.2, and 4.3 shows a schematic representation of the *ABCC2, ABCC4, and ABCC10* genes.

Table 4.1: *ABCC2, ABCC4,* and *ABCC10* subfamily common (minor allele frequency > 1%) variant alleles in different ethnic groups and functional consequences relevant to Tenofovir disoproxil fumarate metabolism.

Gene (alleles)	**Chromosome**	**Exon/Intron**	**Nucleotide change**	**Protein variation**	**Risk of KTD**	***MAF in different populations (%)**		
						Caucasians	Asian	Mixed
ABCC2								
24CC (rs717620)	10	Exon 1	24C>T	--	Increased	0.81	0.79	0.89
1249 G>A (rs2273697)	10	Exon	*1249 G>A,*	Val417Ile	Increased	--	0.046	0.42
2366 C>T, rs56220353	10	Exon	2366 C>T	Ser789Phe	--	--	--	--
3563 T>A (rs17222723)	10	Exon	3563 T>A	Val956Glu	Decreased	--	--	0.01
2934 G>A (rs3740070)	10	--	2934 G>A	Ser978Ser	--	0.01	0.07	--
ABCC4								
669 C>T (rs899494)	13	Exon	*669 C>T*	Arg144Cys	No association	0.83	0.75	---
3463 A>G (rs1751034)	13	Exon	3463 A>G	Lys1116Lys	Lower TDF clearance	0.85	0.67	--
4131 A>C (rs3742106)	13	Exon	4131 A>C	--	Lower TDF clearance	0.47	0.47	--
ABCC10								
526G>A (rs9349256)	6	Exon	526G>A	--	Decreased	0.44	0.6	--
2759T>C (rs2125739)	6	Exon	2759T>C	Ile948Thr	Increased	0.25	0.1	--

** MAF: minor allele frequency. Data compiled from (References (107, 108, 109, 110). Insufficient data is indicated by a hyphen*

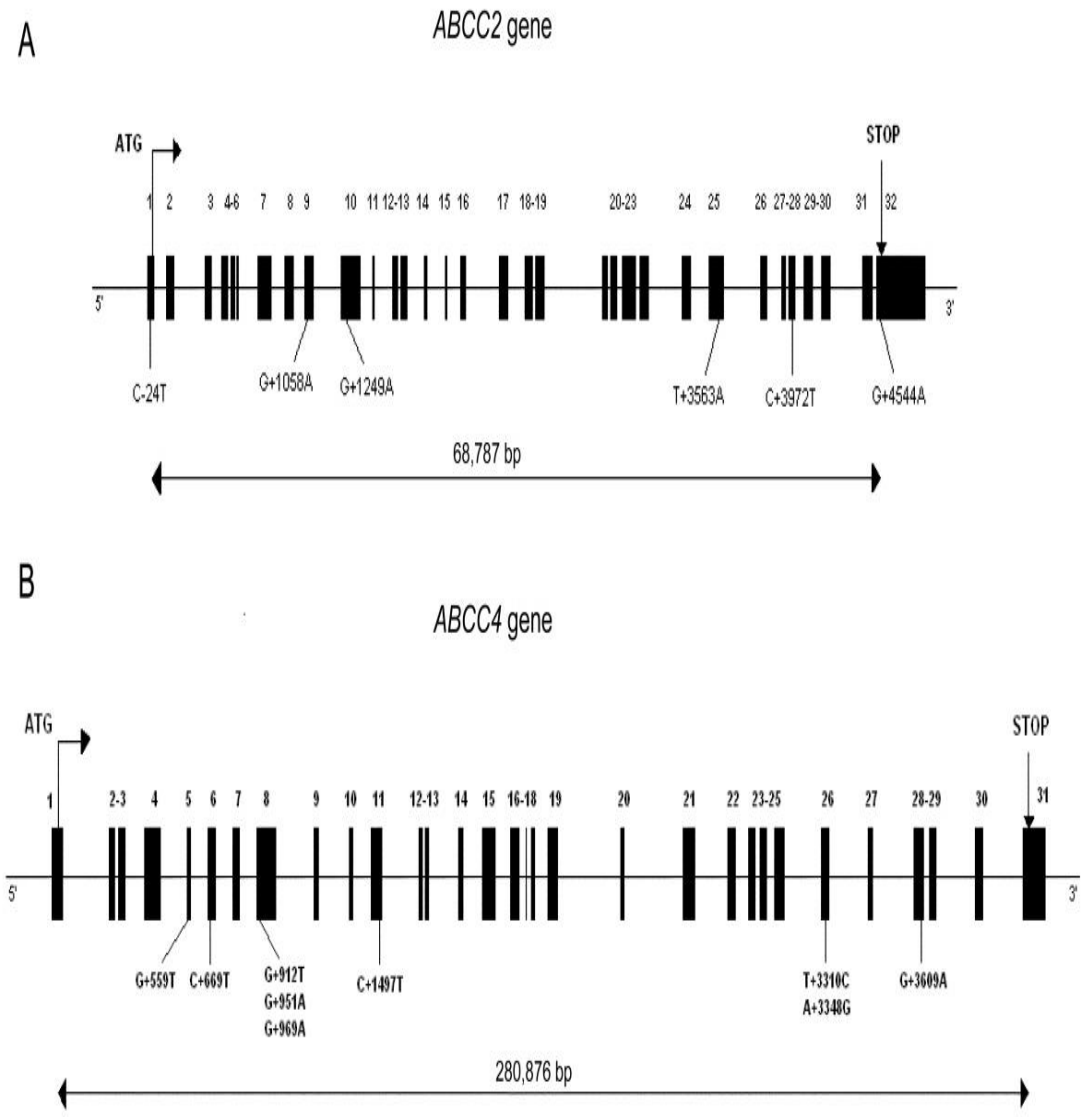


Figure 4.2: Schematic representation of polymorphisms of the *ABCC2* and *ABCC4* genes in HIV positive patients on Tenofovir disoproxil fumarate [adapted from *Izzedine et al*] (109)

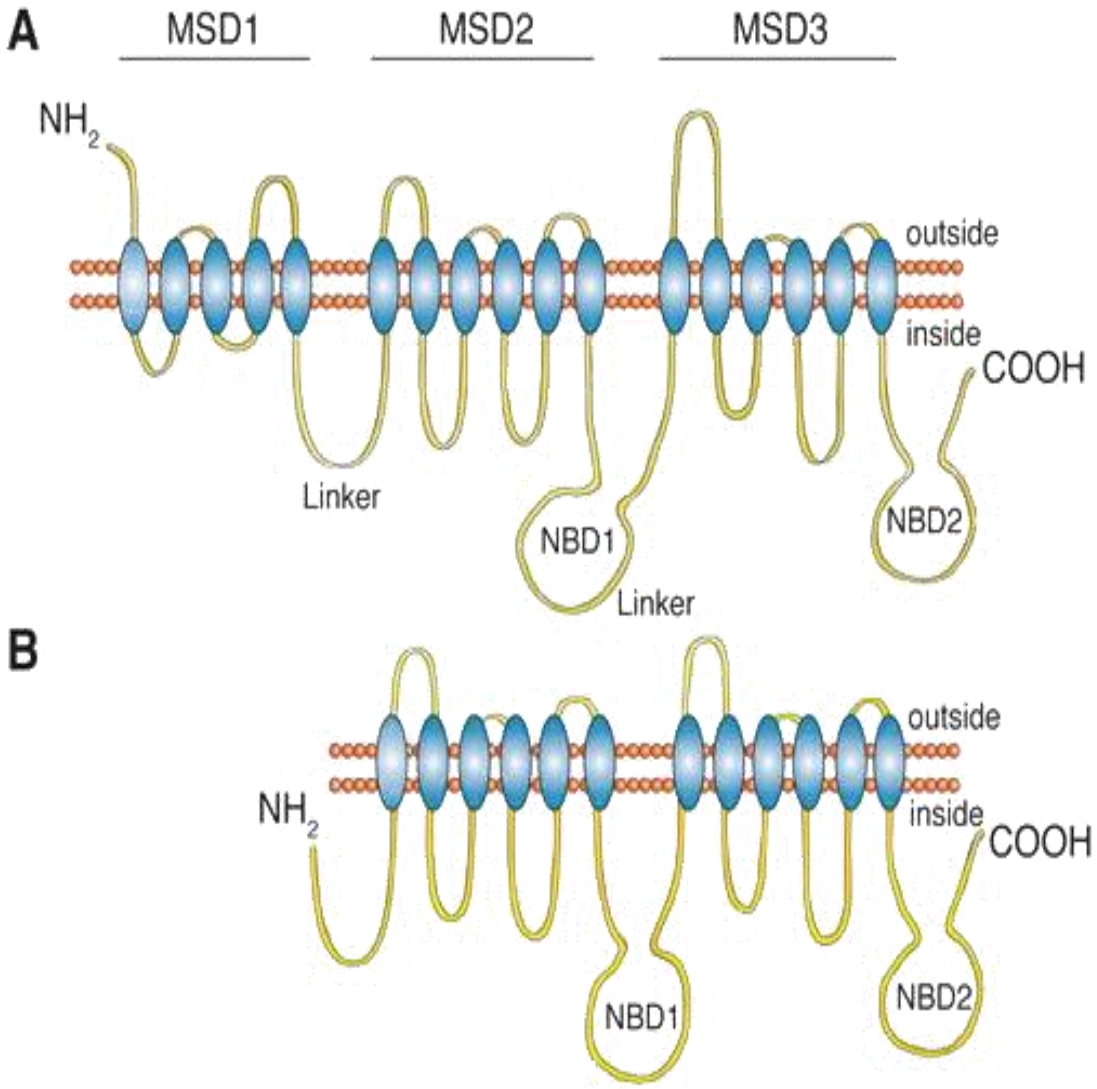


Figure 4.3: A schematic representation of the *ABCC10* gene [adapted from *Hopper et al*] (195)

4.2 Pharmacogenetics of TDF induced kidney tubular dysfunction

Despite increasing reports of association between TDF exposure and risk of KTD, uncertainty remains as to the most appropriate diagnostic modality for adjudication of KTD. In recent studies (107, 108, 110), KTD has been defined by a composite of urinary and serum parameters that perhaps highlight the variable nature of TDF/KTD clinical phenotype, and thus preclude easy applicability of these composite markers in clinical practice. There has been increasing interest from several reports regarding the potential diagnostic utility of LMWP in patients with clinical and sub-clinical

forms of TDF induced KTD (162). Retinol-binding protein (RBP) is a low molecular weight (LMW) protein that is excreted in increased amounts in patients with KTD (162). In the pivotal ASSERT study, patients randomised to TDF experienced significant increases in urinary RBP excretion (196). Additionally, in a cross sectional report, Campbell et al showed that patients exposed to TDF (when administered with a ritonavir-boosted protease inhibitor) were more likely to have substantially elevated urinary RBP concentrations (162). Whether KTD as defined by LMW proteinuria correlates with SNPs of genes encoding transport proteins involved in the disposition of TDF is unknown. I examined association between adjusted RBPCR (as a marker of kidney injury) with SNPs of *ABCC2*, *ABCC4*, *ABCC10*, *SLC22A6* and *SLC22A8* genes in HIV positive patients exposed to TDF in this hospital cohort.

4.3.0 Study design and patient recruitment

4.3.1 Study aims

This study principally investigated the impact of specific SNPs of genes encoding proteins involved in TDF transport, and risk of KTD in HIV positive patients exposed to it. These include SNPs of *ABCC2* (MRP2), *ABCC4* (MRP4), and *ABCC10* (MRP7). These SNPs were selected based on their minor allele frequencies (*www.pharmgkb.org;* *www.cypallelles.ki.se*) and reported associations with KTD from earlier studies amongst other factors (107, 108, 110).

4.3.2 Study design

HIV positive patients attending King's College Hospital, London, United Kingdom between August 2006 to August 2007 were invited to participate in a cross sectional study to examine the prevalence of kidney disease and its associated factors.

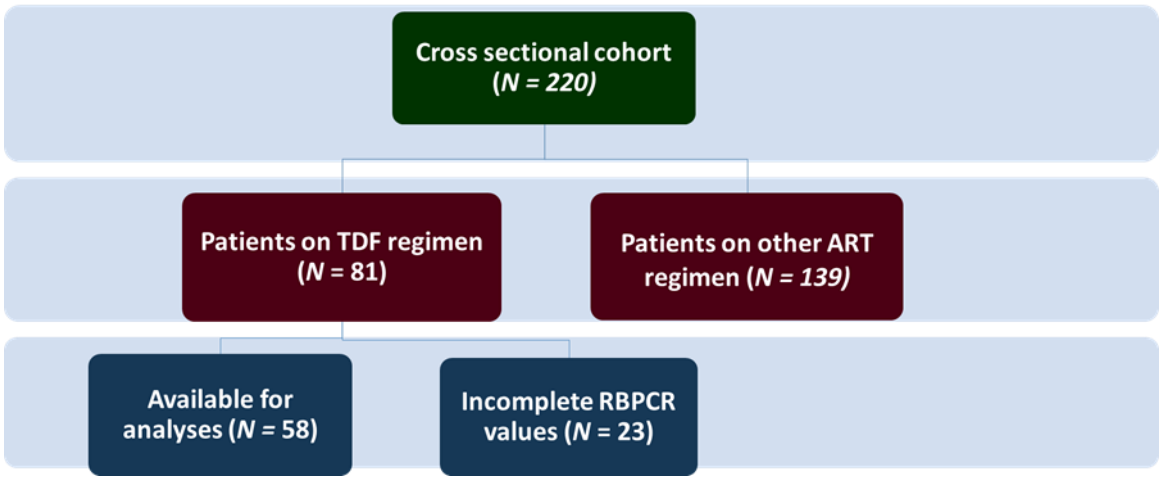


Figure 4.4: Study design and patient enrolment (King's college cohort)

4.4.0 Patient enrolment, screening and sampling

Patients who provided consent and agreed to participate in the study were initially screen and enrolled into the study cohort. A single blood (10mls) and urine (10mls) samples were collected and stored at -70°C until completely used.

4.4.1 Patient recruitment

4.4.2 Inclusion criteria

- Age ≥18 years
- HIV positive "on" or "off" ART drugs
- Any CD4 count or HIV viral load
- Able to give informed consent

4.4.3 Exclusion criteria

- Inability to give informed consent

4.4.4 Case definition

In this study kidney injury (as a standard for subsequent comparison with other novel biomarkers including LMW proteinuria) was defined as:

- eGFR <60mls/min/1.73m^2
- Urine PCR ≥20mg/mmol (this definition was based on the work of Campbell et al (162) in a cross section of HIV positive patients attending outpatients clinic at King's College Hospital London)
- RBPCR ≥ 17μmol/mmol

4.4.5 Ethics and data management

The protocol for the initial cross sectional study was reviewed and subsequently approved by NHS research ethics committee. For this subsequent pharmacogenetic sub-study, an amendment was sent to North Manchester research ethics committee for approval to carry out additional genetic analyses on the repository serum samples. Following review of this amendment, approval was granted for commencement of pharmacogenetic assays and further analysis of stored serum samples.

4.5.0 Genetic analyses

To ensure reliability of the process, and validity of our results, separate DNA processing steps were carried out in designated areas in keeping with best laboratory (lab) practices. Sample preparation was carried out in a category II lab, whilst DNA extraction was done in a designated area of our lab earmarked for nucleic acid work. Preparation of PCR reaction plates including genotyping was carried in a category III

lab under a safety hood in keeping with local GCP and manufacturers' protocol. All DNA work was carried out in the molecular labs of the University of Liverpool

4.5.1 DNA extraction

Genomic DNA was extracted from stored serum samples using the QIAamp DNA extraction kit. This was to allow rapid purification of DNA from our serum sample. I carried this out as follows:

- Frozen serum samples were thawed by placing in preheated water bath for one hour.
- 20µl of QIAGEN protease provided from the manufacturer was pipette into a 2ml Eppendorf to ensure lysis of DNA in the sample.
- 200µl of thawed serum sample was added into this.
- 200µl of AL buffer was added to this mix
- The resulting mixture was pulse vortexed for 15 seconds (s) to ensure adequate mixing of the constituents (buffer, sample, and QIAGEN protease)
- I incubated the mixture at 56^{o} C for 10 minutes. This represents the optimal time for DNA yield, as longer incubation periods have no impact on quality of DNA.
- The mixture was centrifuged at 1000 rotations per minute (RPM)
- 200µl of 96% ethanol was pipette into the mix, and pulse vortexed for 15s to remove drops from the lid.
- I then pipette out the mixture unto a QIAamp Mini spin column (provided with 2mls collection tubes), without wetting the rim of the column. This was centrifuged at 8000 RPM for purification involving four steps below.
- The QIAamp spin column was transferred unto a clean 2mls collection tubes, and the old collection tubes containing the filtrate was discarded

- Centrifuge again at 8000RPM for 1 minute (min)
- I carefully opened the QIAamp spin column and pipette in 500µl of AW1 buffer with adequate care taken not to wet the inside of the rim of the spin column. With the spin column cover closed, it was centrifuged at 8000RPM for 1 min.
- The QIAamp spin column was transferred into a clean 2ml collection tube, with the one containing the filtrate discarded.
- I then opened the lid of the QIAamp spin column and added 500µl of AW2 buffer (took adequate care not to wet the inside of the rim of the spin column). With the lid closed, the spin column columns were centrifuged at 12000 RPM for 1min to ensure all residual AW2 buffer is extruded into the collection tubes.
- With the collections tubes discarded, the QIAamp spin column was transferred into a clean 1.5ml micro-centrifuge tube. 200µl of AE buffer was then added and incubated at room temperature for 5min (to increase DNA yield, considering that we were utilizing serum samples rather than whole blood, and therefore needed to maximize our DNA yield).
- The QIAamp spin column was centrifuged at 8000 RPM for 1min.
- Extracted DNA was stored in -25^{o}C fridge prior to, and during genotyping.

4.5.2 Quantification of DNA

DNA concentration of individual samples was estimated through absorbance at 250nm using spectrophotometer (NanoDrop® spectrophotometry).

The spectrophotometer was initially calibrated with distilled water and DNA concentration. Extracted DNA concentration for individual samples as estimated are given in appendix C)

4.5.3 Genotyping individual SNP's

Made to order primer-probe mix were utilised for genotyping of the seven identified SNP's highlighted earlier. This was made up of sequence-specific forward and reverse primers designed to amplify the SNP sequence of note (table 4.2). Additionally it contains 2 Taqman® MBG probes with NFQ:

1. One VIC® labeled probe (to detect allele sequence 1)
2. One FAM™ labeled probe (to detect allele sequence 2)

Table 4.2: A summary of the VIC/FAM allele context sequences explored

SNP ID	VIC-labeled context sequence	FAM-labeled context sequence
ABCC2 24CC (rs717620)	ACAATCATATTAATAGAAGAGTCTT[**C**]	**[T]**GTTCCAGACGCAGTCCAGGAATCAT
ABCC4 3463A>G (rs1751034)	TGCATACCTGAGGTATGATTGACAT[**C**]	**[T]**TTCTTCCTTAAATCGTGAAGTCCAA
ABCC4 (rs899494)	CCATCCAGAGTAGGGCAGTCACTGC[**A**]	[**G**]ATCGCCTGCAGTGGTCCTGCCCACA
ABCC10 (rs9349256)	GAGTTTTCACTCTCTCCTGACCTTT[**A**]	**[G]**TCCAACCCTGTGCCCCACAGCTCAA
ABCC10 (rs2125739)	ACAGCCCCCTCCTCACCACCCAGCA[**C**]	**[T]**CCCAGTGTTCCCACTGCCCAAAGCT
OAT1 (relative to accession numberAJ249369	CCCACTCGGGCCATGGTGCTGCCCA[**C**]	[**T**]TCCCATGCCTGTCTGCCTGCAGGGC
OAT4 (rs11231809)	AATTCATTCATTCAACAAACATGTA[**A**]	**[T]**TGAGTCCTTACAAACGATACGTCTG

4.5.3.1 Preparation PCR reaction Mix

Table 4.3 gives a summary of the PCR reaction constituents and volume

- Frozen pre-prepared made-to-order primer-probe mix was thawed by placing on ice. Fully thawed, it was further re-suspended by vortexing and centrifuging at 3000 RPM for 1 min
- To minimize the usual freeze-thaw cycles, the 80X cDNA primer-probe mix was diluted with 1X TE buffer (composed of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, in DNase-free sterile filtered water) to a 20X working stock. This was subsequently aliquoted into 1.5ml micro-centrifuge tubes and sored at -25^{o}C.
- I utilised the 96-well plate for running PCR reaction for each SNP, I therefore ensured that the total reaction volume was 25µl. This was made up of 12.5µl of the 2X TaqMan® Master Mix, 1.25µl of 20X made-to-order assay Working Stock, and nuclease free water. Total volume per well was 5µl.

Table 4.3: Summary of PCR reaction constituents and volume

Reaction reagent/DNA	Volume (µl)
ABgene master mix	12.5 X number of samples
Primer probe mix	12.5 X number of samples
Sigma water	8 X number of samples
cDNA	2

4.5.3.2 Preparation of the reaction plate

- 5µl of the reaction mix was pipette carefully into each well (60 of the 96-well plate). We avoided the outer wells to reduce the risk of contamination (see figure 4.5 for plate setup).
- Reaction plate was carefully sealed with a MicroAmp® Optical adhesive film, taking care to expel air bubbles.
- I centrifuged the plate at 1000 RPM for 1 min to further spin down the contents of each well and expel air bubbles.
- The plate was loaded into a PCR cycler (Opticon® 2 DNA engine, version 3.1 Bio-Rad laboratories, Inc., Hercules, Ca., USA) utilising standard methodology. This was set at 95ºC for 15mins, then 40 cycles of 95ºC, and 60ºC for 1min. Following upload of the 96-well reaction plate, preparation of reaction in the PCR machine was done as follows:
- A reaction file is opened on the accompanying Opticon® LCD monitor
- The corresponding FAM/VIC dyes are confirmed and one blank removed.

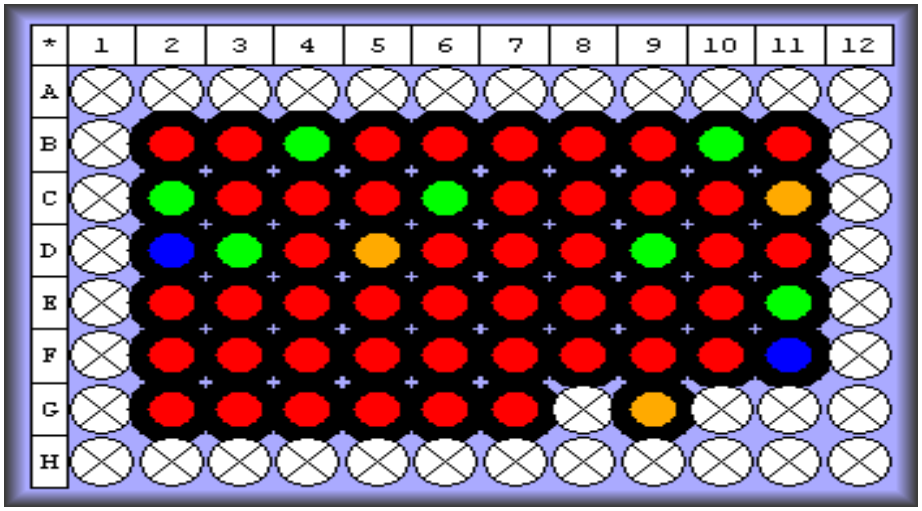


Figure 4.5: PCR reaction plate setup depicting avoidance of the outer wells to reduce risk of sample contamination

4.5.3.3 Reading the plates

Sigmoid curves generated by allelic discrimination of the PCR process, and relevant VIC and FAM dye disposition of the SNP's on the PCR curve were "called" as homozygous, heterozygous, or wild type (Figures 4.6, and 4.7). The excel files containing raw "called" VIC and FAM dye disposition of various SNPS are given in appendix D)

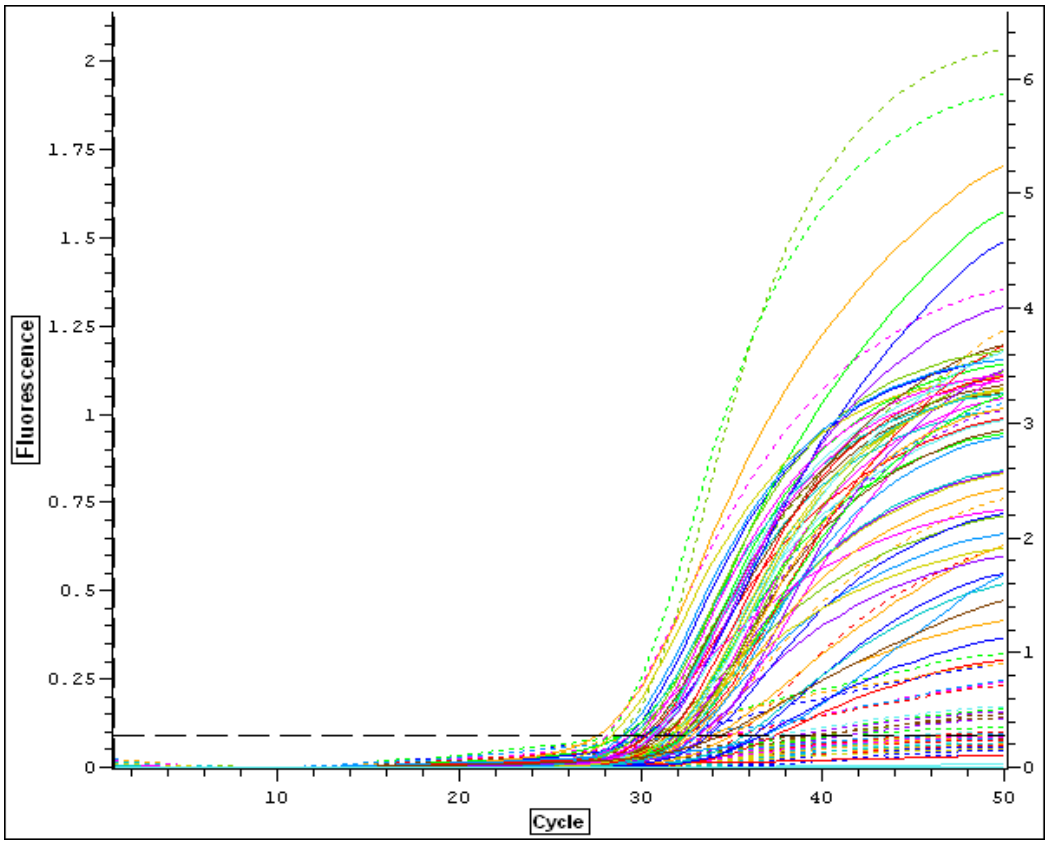


Figure 4.6: Sigmoid amplification plots showing florescence for different study samples. I set the threshold above the detection limit, but above the plateau phase during which amplification slows down according to manufacturer's protocol

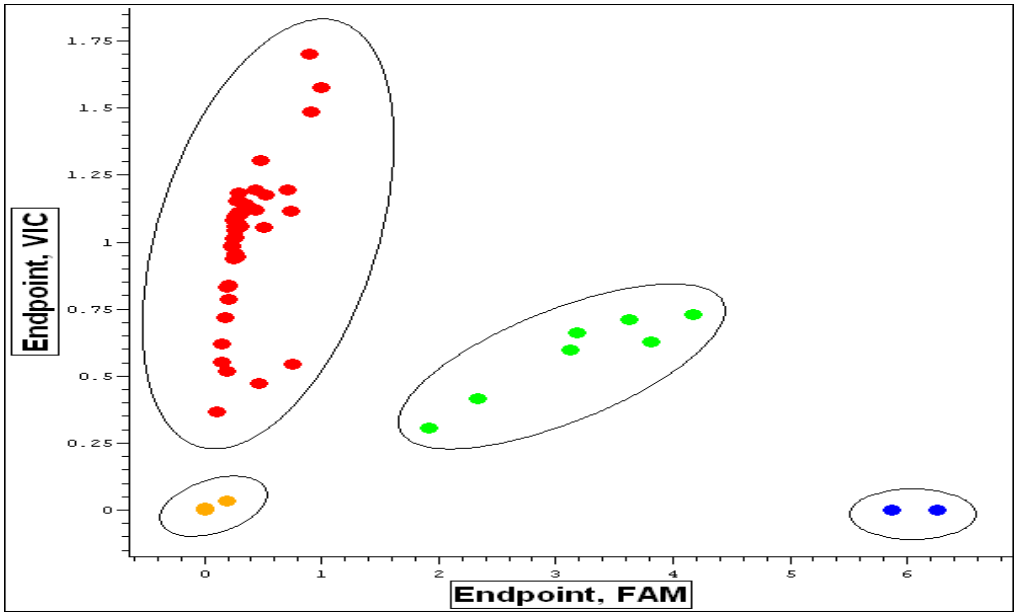


Figure 4.7: Clustering of "called" SNP PCR amplification data into, blanks (orange), homozygous (red), heterozygous (green), and wild type (blue)

4.5.4 Selection of single nucleotide polymorphisms and genotyping

I selected seven SNPs for mutational screening of genes encoding transport proteins involved in the disposition of TDF. The selected SNPs were considered this study based on their previously reported association with risk of KTD from earlier studies [(107, 108, 110) as well as a minor allele frequency of > 5% in the general population. Selected SNPs for analysis in this study includes *ABCC2 24CC* (MRP2; rs717620), *ABCC4* 3463 (MRP4; rs1751034), *ABCC4* 669 (MRP4*;* rs899494*)*, *SLC22A8* (OAT4; rs11231809), *SLC22A6* (OAT1; accession number AJ249369), and *ABCC10* (MRP7; rs9349256, rs2125739).

4.6.0 Statistical analysis and Bio-analytical methods

4.6.1 Statistical analysis

Genotypic frequencies in the study population were compared by Fisher's exact test. All SNPs were tested for deviation from the Hardy-Weinberg equilibrium (HWE) by

Chi squared test using Haploview software (Broad Institute, Cambridge, Massachusetts, USA). Both bivariate and multivariate analyses were carried out to identify predictor variables. Co-variates with $p<0.1$ in univariate analysis were entered into multivariate logistic regression models. P values <0.05 were considered statistically significant. All other data analyses were carried out using StatsDirect version 2.7.9 (StatsDirect Ltd, Altrincham, Cheshire, UK).

4.6.2 Additional bio-analytical methods

Retinol binding protein (RBP) was quantified by enzyme-linked immunosorbent assay (ELISA; Immundiagnostik, Bensheim, Germany; reference range 0.01-0.54 mg/L) and expressed as ratio to creatinine concentration (RBPCR). Urinary RBP corrected for creatinine (mg/mmol) excretion were estimated by laboratory staff at King College Hospital laboratories, London. KTD was defined by an upper quartile RBPCR (>17 µg/mmol) (162). This definition was based on the seminal work of Campbell et al in a cross sectional analysis exploring diagnostic utility of various urinary biomarkers (162).

4.7.0 Results

4.7.1 Patient characteristics

Eighty-one (81) of the 220 patients in this cross sectional cohort received TDF at the time of sampling. Of these, 23 had missing RBPCR values and were not included in analyses. Fifteen (25.9%) of the remaining 58 patients had KTD (as defined by RBPCR ≥17 µg/mmol), whilst the remaining 43 (74.1%) patients served as controls. The median duration of TDF exposure was 583 days. Patients with KTD had higher current CD4 cell counts, lower estimated glomerular filtration rates (eGFR), and higher albumin creatinine ratios (ACR), this is shown in Table 4.4.

Table 4.4: Demographic and clinical characteristics of the study population (N = 58)

Characteristics		**All patients**	**KTD [a]** ***(N =15)***	**Controls** ***(N = 43)***	***P***
Age (years)	*Mean (±SD[b])*	42.2 (±8.2)	46 (±8.3)	41.3 (±8.5)	0.06
Gender (Male)	*N (%)*	60 (73.2)	12 (80)	31 (72.1)	0.55
Ethnicity (White)	*N (%)*	41 (50)	10 (66.7)	19 (44.2)	0.079
Hepatitis C co-infection (Positive)	*N (%)*	7 (8.5)	1 (6.7)	5 (11.6)	0.57
CD4 cell count (cells/mm^3)	*Median (IQR[c])*	398 (246, 526)	491 (417, 598)	361 (227, 492)	**0.006**
HIV RNA (copies/ml)	*Median (IQR)*	50 (50, 50)	50 (50, 50)	50 (50, 50)	0.59
Duration on TDF (Days)	*Median (IQR)*	583 (195, 1035)	794 (210, 1370)	576 (175, 1022)	0.35
Co-exposure to protease inhibitors (PI)	*N (%)*	29 (35.4)	8 (53.3)	13 (30.2)	0.11
Weight (Kilograms)	*Median (IQR)*	73 (66, 81.8)	76.7 (53, 23)	74.4 (55, 121)	0.57
Hypertension (Yes)	*N (%)*	6 (7.3)	2 (13.3)	4 (9.3)	0.75
eGFR (ml/min/1.73m^2)	*Median (IQR)*	85.7 (75.5, 94.6)	78.2 (50.6, 87.9)	88.9 (77.8, 100.7)	**0.006**
Urine PCR[d] (mg/mmol)	*Median (IQR)*	9.4 (6.9, 15.9)	11.2 (6.9, 21.1)	11.1 (1.8, 31.9)	0.112
Urine ACR[e] (g/mmol)	*Median (IQR)*	1.1 (0.7, 2.1)	2.8 (0.16, 12.6)	1.35 (0.094, 6.31)	**0.019**
Serum phosphate (mmol/L)	*Median (IQR)*	0.96 (0.84, 1.1)	0.92 (0, 1.35)	0.98 (0.65, 1.36)	0.09
$TmPO_4/GFR$[f]	*Median (IQR)*	1.0 (0.9, 1.1)	80.8 (62.4, 94.6)	87.8 (66.9, 98.3)	0.08

Data presented are median (interquartile range) for quantitative variables, and number of patients (percentage) for qualitative variables as appropriate. a: Based on RBPCR > or < 17 µg/mmol; b: Standard deviation; c: Inter-quartile range; d: Urine protein creatinine ratio; e: urine albumin creatinine ratio; tubular maximum capacity for renal phosphate re-absorption to glomerular filtration rate (GFR) ratio

4.7.2 Association of KTD with single nucleotide polymorphisms of genes encoding TDF transport proteins

The distribution of genotypes of various SNPs is shown in Table 4.5. All evaluated SNPs achieved HWE (<0.05). There was a lower expression of the genotype *CC* at position 24 of the *ABBC2* (MRP2, rs717620) gene in patients with KTD compared with *CT* and *TT* genotypes (odds ratio [OR] 0.29; 95% confidence interval [CI] 0.08-0.96, p= 0.04), Figure 4.8. There was a trend towards reduced risk of KTD with the intronic *G* allele of *ABCC10* (MRP7, rs9349256; OR = 0.4, 0.2-1.0, P = 0.08), and increased risk with the A allele of the influx transporter gene *SLC22A11* (OAT 4, rs11231809, OR 2.3, 0.9-5.8, P = 0.07).

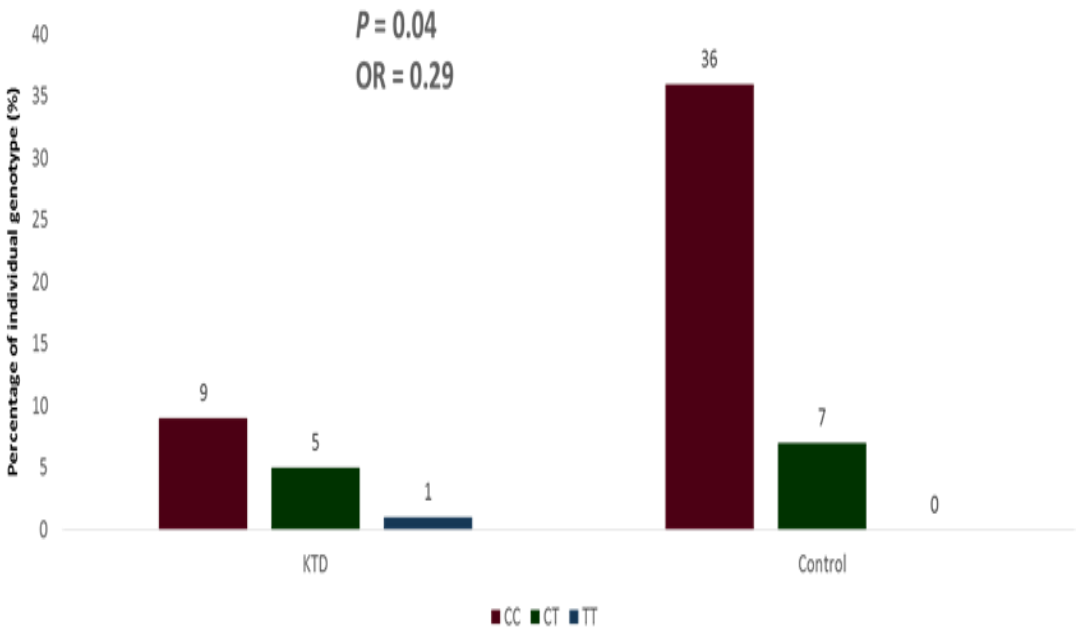


Figure 4.8: Graphical representation of the *ABCC2 24CC* (rs7171620) allele frequencies in the study cohort

Table 4.5: Allelic frequencies of single nucleotide polymorphisms of genes encoding Tenofovir transport proteins in HIV positive patients with and without kidney tubular dysfunction (KTD[a])

SNP[b] ID	Patients with KTD [a] *N* = 15	Controls *N* = 43	*OR (95% CI)*	*P*-value
ABCC2 24 (rs717620) MRP2 (Chromosome 10)				
Homozygous variant TT	1 (6.7%)	0		
Heterozygous CT	5 (33.3%)	7 (16.3%)		
Homozygous wild type CC	9 (60.0%)	36 (83.7%)		
C	23 (76.7%)	79 (91.9%)		
T	7 (23.3%)	7 (8.1%)		
ABCC4 3463 (rs1751034) MRP4 (Chromosome 13)				
Homozygous wild type TT	8 (57.1%)	26 (60.4%)		
Heterozygous CT	3 (21.4%)	15 (34.9%)		
Homozygous variant CC	3 (31.4%)	2 (4.7%)		
T	19 (67.9%)	67 (77.9%)		
C	9 (32.1%)	19 (22.1%)		
ABCC4 669 (rs899494) MRP4 (Chromosome 13)				
Homozygous wild type CC	--	--		
Heterozygous CT	3 (20%)	10 (23.3%)		
Homozygous variant TT	12 (80%)	33 (76.7%)		
C	3 (10%)	10 (11.6%)		
T	27 (90%)	76 (88.4%)		
SLC22A11 (OAT 4) rs11231809 (Chromosome 11)				
Homozygous wild type TT	8 (53.3%)	30 (69.8%)		
Heterozygous AT	3 (20%)	9 (20.9%)		
Homozygous variant AA	4 26.7%)	4 (9.3%)		
T	19 (63.3%)	69 (80.2%)		
A	11 (36.7%)	17 (19.8%)		
SLC22A6 453 GA (OAT1) [c] (Chromosome 2)				
Homozygous wild type CC	12 (80%)	29 (67.4%)		
Heterozygous CT	3 (20%)	12 (27.9%)		
Homozygous variant TT	0	2 (4.7%)		
C	27 (90%)	70 (81.4%)		
T	3 (10%)	16 (18.6%)		
ABCC10 (rs9349256) MRP7 (Chromosome 6)				
Homozygous variant AA	7 (46.7%)	8 (19%)		
Heterozygous AG	3 (20%)	17 (38.1%)		
Homozygous wild type GG	5 (33.3%)	18 (42.9) %		
A	17 (56.7%)	33 (38.4%)		
G	13 (43.3%)	53 (61.6%)		
ABCC10 (rs2125739) MRP7 (Chromosome 6)				
Homozygous variant TT	6 (54.5%)	9 (29.0%)		
Heterozygous CT	3 (27.3%)	17 (54.8%)		
Homozygous wild type CC	2 (18.2%)	5 (16.1%)		
T	15 (68.2%)	39 (62.9%)		
C	7 (31.8%)	23 (37.1%)		

Note: Data are number of Alleles/Genotype with percentages in bracket a: As defined by RBPCR >17 µg/mmol; b. Single nucleotide polymorphisms; c. Relative to accession no: AJ24936

4.7.3 Diagnostic performance of retinol binding protein/creatinine ratio (RBPCR)

I generated AUC/ROC curves to evaluate the performance of upper quartile RBPCR (>17μg/mmol), adjusted NGAL/CR, serum creatinine (mmol/L), and eGFR (mls/min/1.73m^2) in differentiating patients with kidney tubular injury from those with normal kidney function. Utilising urine PCR ≥20mg/mmol as cut-off for kidney dysfunction, AUC-ROC of upper quartile RBP/CR (>17 μ /mmol) was 0.67, (CI = 0.55-0.81, p = 0.01). This is shown in figure 4.9.

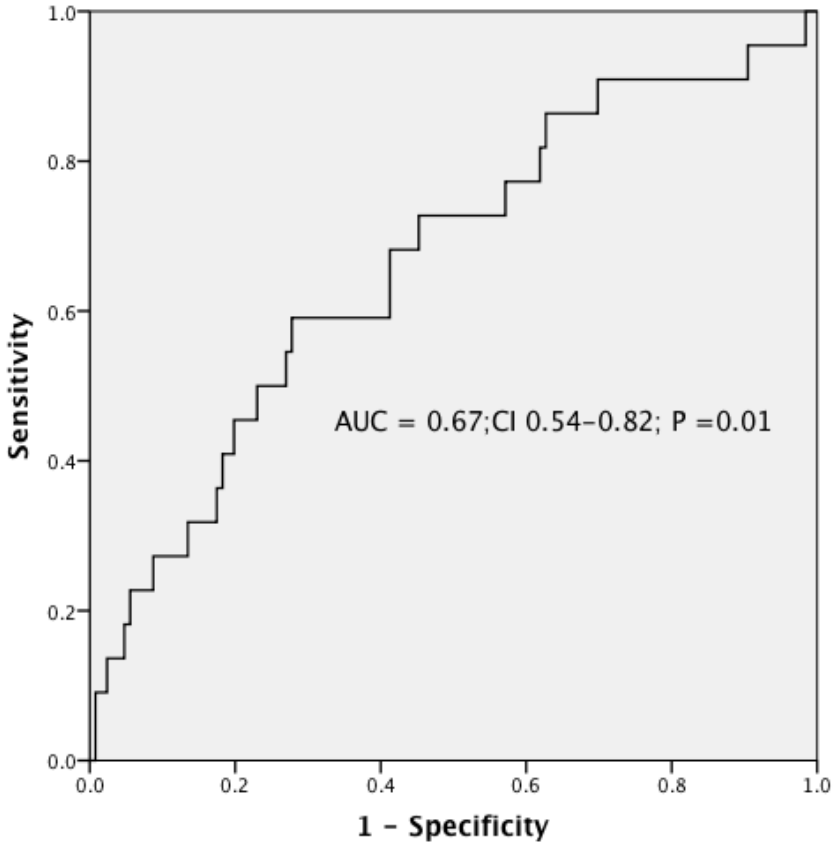


Figure 4.9: AUC-ROC of upper quartile retinol binding protein/creatinine (RBPCR) as a diagnostic marker of kidney injury in HIV positive patients on TDF-based regimen

It is noteworthy that out of the six evaluated surrogate markers of kidney injury, eGFR had the least AUC-ROC (0.37, CI = 0.22-0.53, P = 0.07). Conversely, urine ACR had the highest AUC-ROC compared with other diagnostic markers for kidney injury in these cohorts of patients. RBPCR had the next highest AUC-ROC compared to established surrogate markers of kidney injury. The areas under the curves as well as sensitivities and specificities of other biomarkers are shown in figure 4.10 and table 4.6.

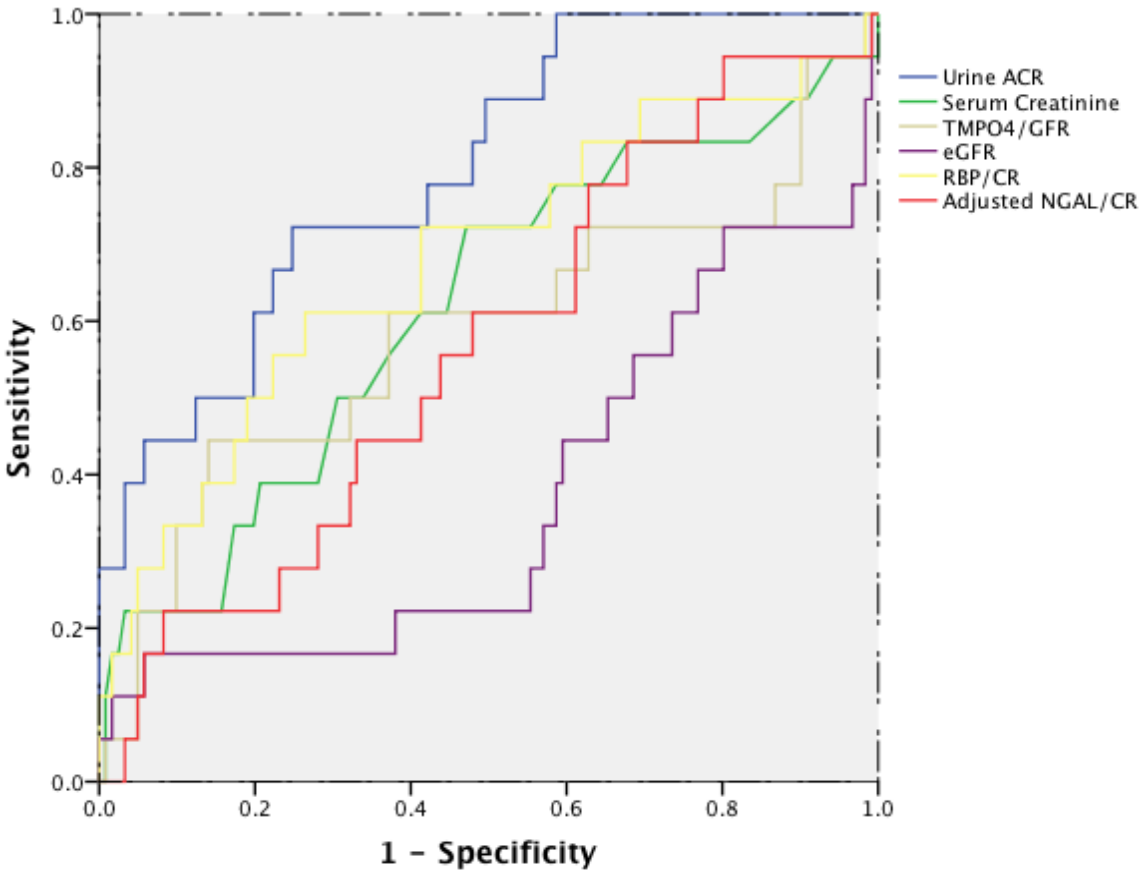


Figure 4.10: ROC curves comparing diagnostic performance of various Kidney biomarkers in HIV positive patients on TDF-based regimen. The more leftward the plots, the higher their reliability as diagnostic markers of kidney injury in these cohorts of patients

Table 4.6: Area under the curve (AUC) and confidence intervals of comparative performance of biomarkers of kidney injury in HIV positive patients exposed to TDF-based regimen.

Kidney biomarkers	***AUC***	***P***	**95% confidence interval**	
			Lower bound	**Upper bound**
Urine ACR (g/mmol) [1]	0.79	0.001	0.69	0.90
Serum creatinine (mmol/l)	0.61	0.105	0.46	0.76
TMPO4/GFR (mmol/L) [2]	0.58	0.246	0.41	0.75
eGFR (mls /min/1.73m^2)	0.37	0.078	0.21	0.52
RBPCR (μg/mmol)	0.67	0.014	0.53	0.82
Adjusted NGAL/Creatinine	0.56	0.366	0.42	0.70

1. ACR; urine albumin creatinine ratio; 2. TMPO4/GFR: Tubular maximum capacity for renal phosphate reabsorption to glomerular filtration rate (GFR) ratio

4.7.4 Independent predictors of kidney tubular dysfunction in HIV positive patients exposed to Tenofovir disoproxil fumarate

Table 4.7 shows factors associated with risk of KTD in HIV positive patients exposed to TDF. Univariate odds ratios were calculated for each of the SNPs of interest. Possession of genotype *CC* at position 24 of the *ABCC2* gene (MRP2, rs717620) was the only SNP significantly associated with reduced risk of KTD. This SNP remained significantly associated with KTD after adjustment for age, and eGFR (adjusted OR = 0.05; 0.003-0.71, P = 0.027)

Table 4.7: Factors predicting risk of kidney tubular dysfunction (KTD) [1] in HIV positive patients exposed to TDF

Parameter	**Univariate analysis**		**Multivariate analysis**	
	OR (95% CI)	***P***	**OR (95% CI)**	***P***
Age (Years)	0.9 (0.99-1.0)	0.07	1.0 (0.91-1.1)	0.53
*ABCC2 24CC (*rs717620) MRP2	Genotype CC 0.24 (0.06-0.93)	0.04	0.05 (0.003-0.71)	**0.027**
ABCC10 (rs2125739) MRP7	C allele 3.0 (0.54-16.8)	0.21	--	--
ABCC10 (rs9349256) MRP7	*G allele* 0.42 (0.1-.1.7)	0.2		
ABCC4 669 (rs899494) MRP4	T allele 0.83 (0.19-3.5)	0.78	--	--
ABCC4 3463 (rs1751034) MRP4	*C allele 2.5 (0.31-20.4)* *T allele 2.1 (0.29-14.7)*	0.39 0.46	--	--
SLC22A11 (rs11231809) OAT4	A allele 0.28 (0.03-1.31)	0.11	--	--
SLC22A6 (453 GA) OAT1 [2]	A allele 1.93 0.46-7.9	0.36	--	--

1. Kidney tubular dysfunction; 2. Relative to accession no: AJ249369

4.7.5 Haplotype analyses

Exploratory haplotype analyses of the *ABCC2-ABCC4* SNPs encoding genes involved in the bio-disposition of TDF, showed no significant association between KTD and any of the tested haplotypes.

4.8.0 Discussion

A number of genes encoding transport proteins involved in the bio-disposition of TDF have been known to be polymorphic (107, 108, 110). This is the first study exploring the potential relationship between SNPs of genes encoding transport proteins involved in the bio-disposition of TDF, and risk of kidney tubular injury in HIV positive patients as defined by LMW proteinuria (RBPCR). Since its approval in 2001, TDF continuous to be widely used and its efficacy and safety has been well established in numerous studies (144, 145). Nonetheless, a small proportion of patients develop severe kidney tubular toxicity (including Fanconi syndrome), with a variable proportion of patients (6-22%) reportedly having subclinical KTD (145, 147, 149). Previous studies have suggested possession of genotype *CC* at position 24 of the *ABCC2* (MRP2, rs717620) gene (107, 108, 109), *ABCC10* (MRP7, rs9349256, and rs2125739) (110) including the extended haplotype *ABCC10-ABCC2* (GGC-CGTC) (110) in addition to age, and low body weight (107) as potential determinants of KTD in these cohorts of patients. In these studies, KTD was defined by a composite of serum and urinary parameters. In this report, I confirm the association between *ABCC2 24CC* and KTD, and observed a non-significant association for polymorphisms in *ABCC10* and *OAT3* genes.

This study finding of reduced KTD risk with the *ABCC2 24CC* (rs717620) SNP is not consistent with reports from previous studies (107, 108, 109). These reports showed increased risk of KTD with possession of this genotype. It is noteworthy

however that these studies utilised a different surrogate marker of kidney tubular injury (discussed elsewhere in this report) than the one used in this study (RBPCR). How the mode of case ascertainment of KTD explains the difference in outcome between this report and previous reports (107, 108, 109) is still uncertain. The novelty of this report is both in its finding of significant association between *ABCC2 24CC* (rs717620) and LMWP-defined KTD, as well as possible protective effect of this genotype in this cohort of patients. In a recent study evaluating association between *ABCC2 24CC* SNP and risk of KTD in a prospective cohort of HIV positive patients on TDF, Nishijima et al (2015) (115) failed to establish any link between possession of this SNP and risk of KTD.

Kidney tubular dysfunction comprises a spectrum with Fanconi syndrome representing the extreme phenotype, and its definition to date has relied on a composite of several urine and serum parameters (107, 108, 110). The use of RBP as a single biomarker provides a convenient way to assess KTD in clinical practice. It is also noteworthy that on further evaluation of the diagnostic performance of various kidney biomarkers (utilising threshold of urine PCR as standard), RBPCR had a high AUC-ROC. This will suggest its potential utility as a surrogate marker of kidney dysfunction in this cohort of patients.

4.8.1 Limitations

Despite its novel attempt, this study is limited firstly, by lack of robust power to demonstrate an independent association with KTD. Secondly, the quality of DNA extracted from serum rather than whole blood may have affected potential associations between the evaluated SNPs and LMW proteinuria. Additionally, a candidate gene-based approach may represent a limited attempt at exploring potential genetic associations compared to genome wide association study (GWAS). With the

latter evaluating the whole genome, thereby highlighting potential associations. Owing to these limitations, I suggest that the findings of this study are interpreted in the context of previous published work in this area.

4.8.2 Conclusion

In conclusion I have demonstrated that possession of genotype *CC* at position 24 of the *ABCC2* (MRP2 rs7171620) gene was associated with reduced risk of KTD (as defined by an elevated RBPCR level) in HIV positive patients exposed to TDF.

CHAPTER FIVE

ASSOCIATION BETWEEN KIDNEY INJURY MOLECULE 1 (KIM-1), AND RISK OF KIDNEY DYSFUNCTION FOLLOWING ANTIRETROVIRAL THERAPY (ART) DRUG EXPOSURE: THE DETIKI CLINICAL COHORT

5.0 Introduction

I set out to conduct a biomarker association study in an outpatient population of HIV positive patients attending clinics in an urban tertiary centre. This is to determine potential association between low molecular weight proteinuria (LMWP), and risk of kidney injury in these cohorts of patients exposed to antiretroviral therapy (ART) drugs.

5.1.0 Methods

5.1.1 Clinical trial design

This study cohort is comprised of two separate sub-studies all recruited from patients attending outpatient HIV clinics at the Royal Liverpool University Hospital (RLUH). Sub-study one is the cross-sectional arm that was scheduled to recruit HIV positive patients (N = 200) "on" ART drugs for cross sectional comparison with HIV positive patients "off" ART drugs (N = 100). See figure 5.1. The second arm is a prospective sub-study that was scheduled to recruit 75 HIV positive patients just starting ART for longitudinal follow-up at designated time points. At the time of analyses, a combined total of 127 patients have been recruited into both studies. Figure 5.1 gives the patient recruitment summary.

5.1.2 Cross-sectional sub-study

All consecutive HIV infected patients either "on" (cases) or "off" (controls) ART drugs that met inclusion criteria for the study and who attended HIV outpatient clinics in designated recruitment centre were approached, counselled, consented, and enrolled in the study. We scheduled to recruit a study cohort of HIV-positive patients (N =200) on ART drugs for cross sectional comparison with a randomly selected control cohort of HIV-positive patients (N = 100) off ART (both ART naive and

previously experienced). However, at the time of analyses, 114 patients have already been recruited into this arm of the study. Figure 5.2 gives a summary of various patients cohorts recruited at the time of analyses. Patients were consented and this includes a separate consent to participate in genetic research. Clinical, demographic, and laboratory parameters were abstracted from patient's case notes and electronic online records.

5.1.3 Prospective longitudinal sub-study

All consecutive HIV infected patients starting ART drugs that met inclusion criteria for the study, and who attend HIV outpatient clinics in designated recruitment centres were approached, counselled, consented, and recruited into the study. Study cohorts of HIV-positive patients (N = 75) starting ART were scheduled to be recruited into this arm of the DETIKI clinical cohort. However owing to problems with logistics and recruitment attrition, the total number of participants enrolled into this arm of the study at the time of analyses were eleven (N = 11). These patients were consented including a separate consent to participate in genetic research (see appendix 1). At screening demographic, clinical, and laboratory parameters were abstracted from patient's case notes and electronic records. In addition, whole blood and random urine samples were collected for the determination of parameters as shown in table 5.1. An observational follow-up of study participants was scheduled at 4 and 24 weeks. At each study week visits, blood and freshly voided random urine samples were collected and stored for the determination of parameters as stated in table 5.1.

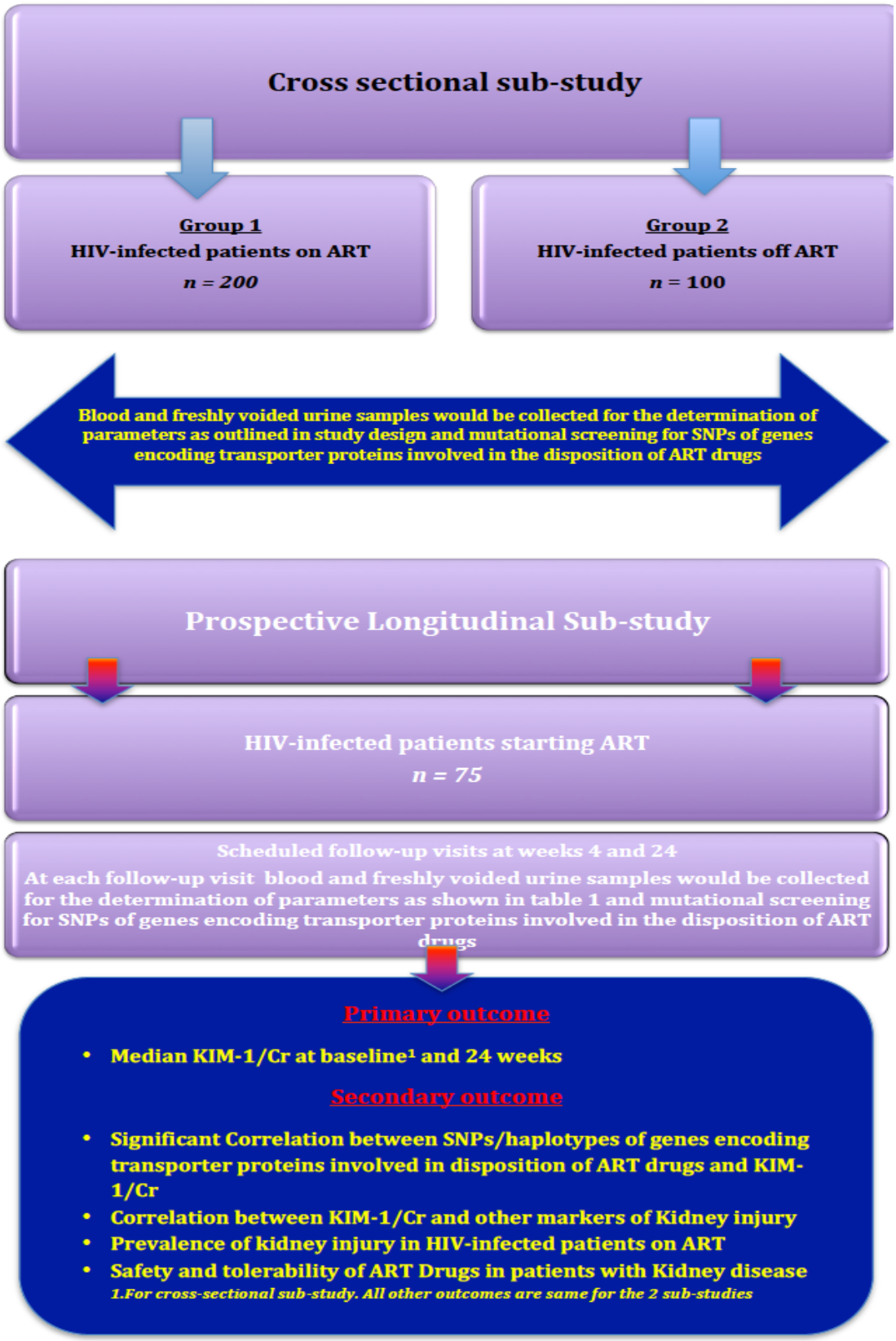


Figure 5.1: Study flow diagram

5.2.0 Clinical trial setting and patient recruitment

Study participants were recruited from HIV outpatient clinics at the RLUH.

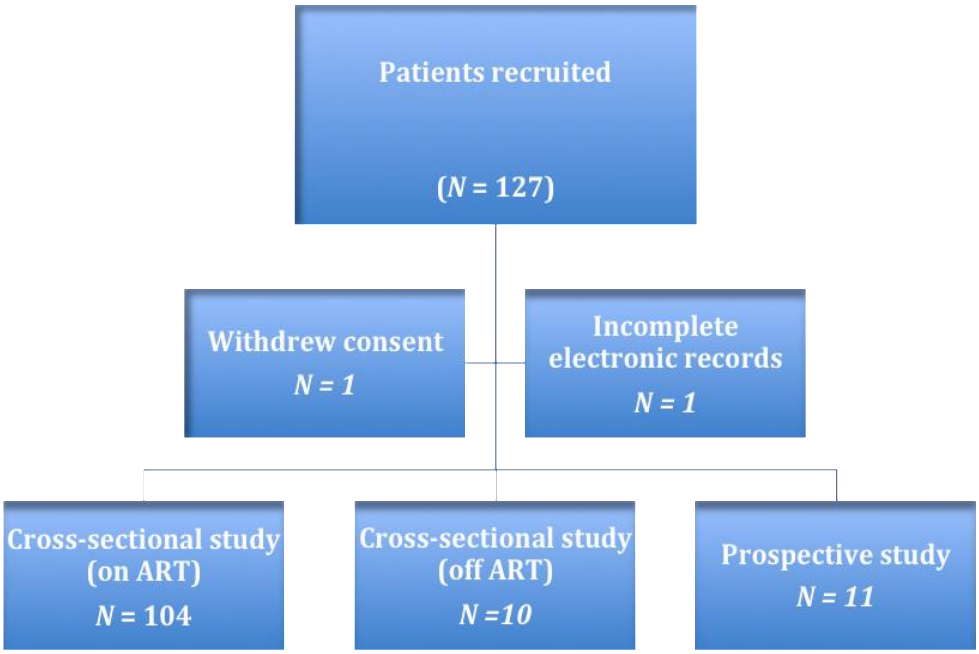


Figure 5.2: Flow chart of DETIKI study cohort showing patient accrual into various study arms

5.3.0 Study population and recruitment sites

Study population were entirely comprised of HIV positive patients that met inclusion and exclusion criteria and who attend HIV outpatient clinics at RLUH.

5.4.0 Definition and adjudication of clinical cases and controls

1. As a standard for comparison, proteinuria in this study is defined as urine protein/creatinine ratio (PCR) ≥20mg/mmol. This was based on seminal work of Campbell et al detailed in previous section of this thesis (162).
2. Exposure to various ART drugs were categorised as ART naive, protease inhibitor (PI) based regimen (ART/PI), Tenofovir/Protease inhibitor based

regimen (TDF/PI), and "other ART regimen" (including NNRTIs, fusion inhibitors, Integrase strand transfer inhibitors).

3. Kidney failure/injury/dysfunction was defined as any one or a combination of the following:

- eGFR <60mls/min/1.73m^2
- Urine PCR ≥20mg/mmol (162)

4. Patients with normal kidney function (as determined from 3 above) were classified as controls.

Table 5.1: Study flow chart (Prospective Longitudinal sub-studies)

Activity	Baseline	4weeks	24 weeks
Medical history	X		
Physical examination	X		
e-GFR[1]	X	X	X
Serum biochemistry [2]	X	X	X
TMPO4/GFR [3]	X	X	X
Urine ACR/PCR[4]	X		X
KTD biomarkers [5]	X		X
Urinalysis	X		
Weight (kilograms)	X		X
Height (meters)	X		
CD4 count/Viral load	X		X
Hepatitis B and C status	X		
Genetic analysis	X		
Informed consent	X		

1. Calculated from simplified 4-variable MDRD equation. 2. Includes Creatinine (Cr), Urea, alkaline phosphatase, liver function test (LFTs), glucose, corrected calcium, inorganic phosphate, uric acid, thyroid function tests (TFTs), serum lipids 3. Tubular maximum capacity for renal phosphate reabsorption to glomerular filtration rate (GFR) ratio. 4. Albumin creatinine ratio/protein creatinine ratio. 5. Kidney injury molecule-1 (KIM-1): Cystatin C (Cys C)· N-acetyl-D-glucosaminidase (NAG): Neutrophil gelatinase-associated lipocalin (NGAL); Fatty acid binding protein (FABP)

5.5.0 Cohort recruitment

5.5.1 Inclusion criteria (cross sectional)

1. Age ≥18 years
2. Any CD4 count or viral load
3. HIV-positive patients “on” or “off" ART drugs
4. Able to provide informed consent

5.5.2 Inclusion criteria (prospective)

1. Age ≥18 years
2. Any CD4 count or viral load
3. HIV-positive patients starting ART drugs
4. Able to provide consent

5.5.3 Exclusion criteria (cross sectional and prospective arms)

Unable to provide informed consent

5.6.0 Sample collection, storage and estimation

For both sub-studies, 10 millilitres (mls) of whole blood and random urine samples (10mls) were collected for the determination of pre-specified study parameters at baseline. Additionally, for patients on the prospective arm at 4 and 24 weeks follow-up, blood and freshly voided random urine samples were collected and stored for determination of parameters as stated earlier (figure 5.1) until completely used. In addition, some of the whole blood sample (5mls) will be stored for mutational screening of SNPs of genes encoding transport proteins involved in the bio-disposition of ART drugs.

5.6.0 Bio-analytical methods

5.6.1 The determination of KIM-1 and urinary creatinine concentration

Following initial introduction to the technique of urinary KIM-1 and creatinine estimations by the Dr. Steve McWilliams (Post-Doctoral Fellow, Wolfson Centre for personalized medicine, University of Liverpool) on an initial trial set of my study samples, I carried out the estimation of the entire cohort of my study samples myself.

5.6.2 Principle of KIM-1 assay

The MSD platform provides for rapid detection and estimation of protein target from a small volume of a given sample. The KIM-1 assay I utilised is a form of MSD platform assay that takes the form of sandwich immunoassay. The MSD plate is provided from the manufacturers (Meso Scale Discovery® [MSD], MD, USA), pre-coated with capture antibody adhered to a working electrode at the base of each well. Into each well, the investigator is required to add the sample of interest (blood, serum, urine, etc.), and a solvent containing detection antibodies tagged with electroluminescence labels (MSD SILFO-TAG™), with varying incubation periods at each stage. KIM-1 (as in our case) or any analyte of interest binds to the capture antibodies adhered to the working electrode. Attraction and binding of labeled (MSD SILFO-TAG™) detection antibodies completes the reaction sequence or "sandwich". The addition of an MSD read buffer provides the chemical milieu necessary for elctrochemiluminescence to be expressed. Loading the MSD plate unto an MSD SECTOR® imager, ensures that when a voltage is applied to the MSD plate electrodes, the captured labeled antibodies "complexed" to the analyte (KIM-1) emits light. The intensity of this light as measured by the imager gives a quantitative estimation of the analyte (KIM-1) in the sample, figure 5.3.

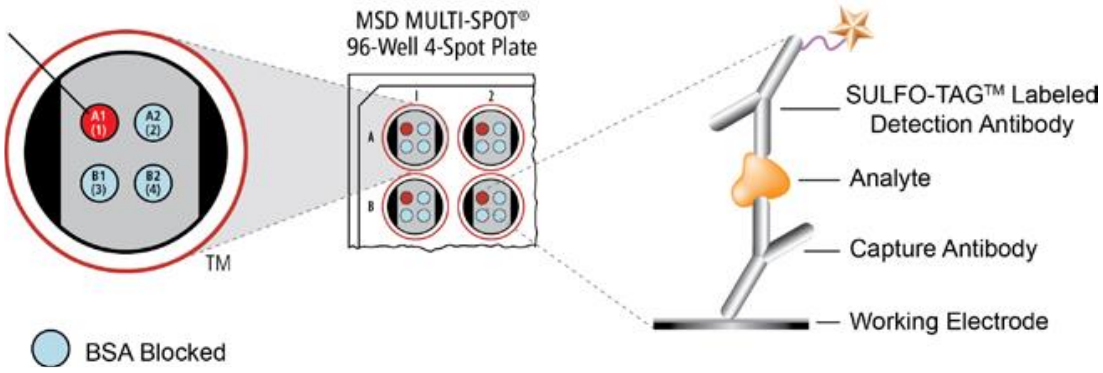


Figure 5.3: Principle underlying KIM-1 assay on MSD platform (adapted from Meso Scale Discovery® [MSD], MD, USA),

As a standard we took the lower limit of quantitation (LLOQ) is greater than two standard deviations above the background value for the assay (which is the concentration of the diluent 37)

5.6.3 KIM-1 assay procedure

Urinary KIM-1 was estimated from stored urine samples according to manufacturers' protocol (Meso Scale Discovery® [MSD], MD, USA), as set out in steps below:

Step one: Preparation of standard calibration curve solution

Stored urine **(at -80ºC)** was thawed, mixed, and centrifuged at 3000 RPM for one minute.
The stock calibrator was thawed on ice and an 8-point standard calibration curve solution was prepared. I prepared calibration standard solution curves for three replicates as follows:

- I initially prepared the highest standard by adding 15µl of stock calibrator to 285µl of diluent 37
- I then prepared the next standard by transferring 60µl of the highest standard to 180µl of diluent 37
- The above process (4 serial dilutions) was repeated five times to generate seven standards (solutions)

- Diluent 37 was used as the 8th blank calibrator (standard)

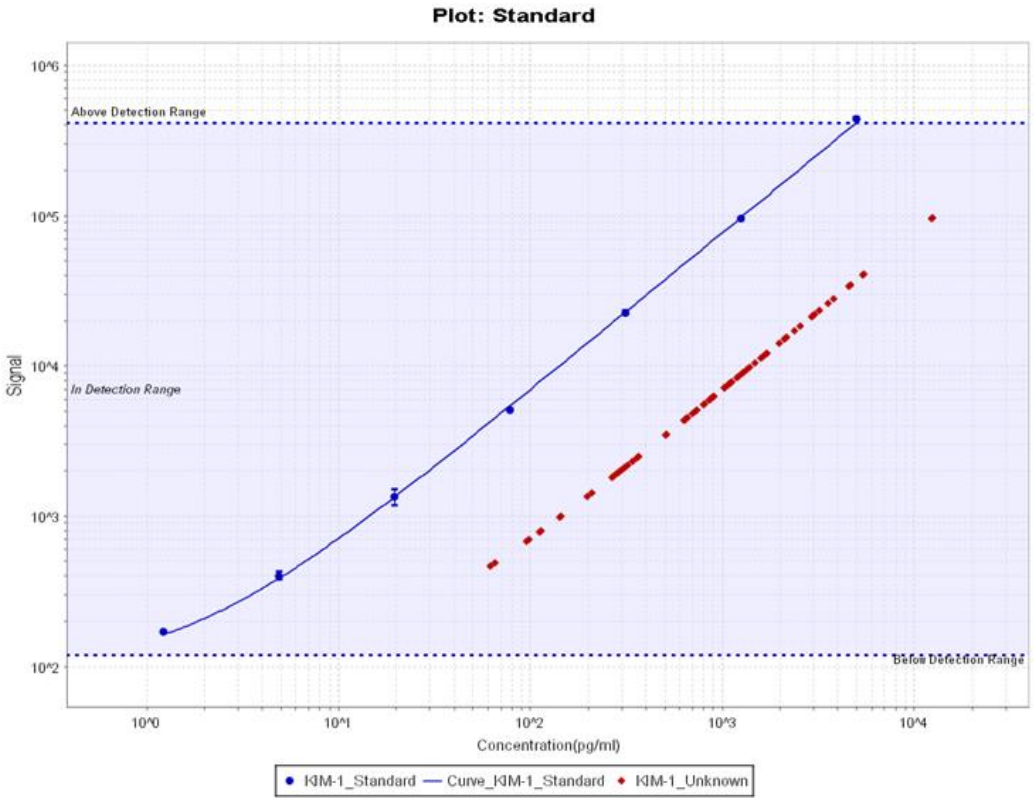
Step two: MSD plates
Manufacturers 96-well MSD plates were supplied pre-coated with capture antibodies (MSD SULFO-TAG^{TM})

Step three: Assay protocol

- I pipetted 150µl of blocker A to each well of the 96-well MSD plate
- I washed the plate 3 times with 300µl/well of PBS-T buffer solution ensuring that there were no residual buffer solutions adhering to the bottom of the wells.
- 50µl of sample solution was added to each well and incubated at room temperature with vigorous shaking (at 1000RPM) for 30mins
- The plate was sealed with adhesive film tightly, and incubated (at room temperature) for 2 hours with vigorous shaking at 700 RPM
- The plate was washed thoroughly with 300µl of PBS-T buffer 3 times
- 25µl of 1X SULFO-TAG^{TM} labeled detection antibody was added to each well, and the plate was sealed with adhesive film and incubated at room temperature for 2 hours with vigorous shaking (700RPM).
- The MSD plate was thoroughly washed with 300µl of PBS-T buffer solution 3 times ensuring that no residual buffer remained in the wells.
- I then added 150µl of 2X read buffer to each well of the 96-well MSD plate
- The plate was analysed on the MSD sector imager.
- MSD DISCOVERY WORKBENCH® software generates a standard curve, which is used to calculate the concentration of KIM-1 in the sample. Accurate quantification of KIM-1 values were obtained by generating a standard curve for each plate utilising atleast 2 replicates from the calibrator (see figure 5.4 for KIM-1 standard and unknown (sample) curves for this study)

5.6.4 Estimation of KIM-1

The upper and lower limits of quantitation (ULOQ, LLOQ respectively) on a seven-point calibration curve were 5000, and 1.22 pg. /ml respectively. Intra and inter-assay precision were <6, and <7% respectively, on three plates on different occasions with recovery of spiked samples at 104–107%. Dilutional linearity was shown at 1:10, 1:100, and 1:1000 dilutions in the assay diluent. The LLOQ was about two standard deviations greater than the background value of the diluent alone, the LLOQ was taken as the limit of detection. Subsequently derived KIM-1 values were normalized to urinary creatinine **(**determined spectrophtometrically) and expressed as KIM-1/Cr (ng/mg).



.

Figure 5.4: Standard and DETKI cohort KIM-1 estimated concentrations

5.6.5 Estimation of urinary creatinine

Urinary creatinine was estimated initially by preparation of seven-creatinine curves standard solution in keeping with manufacturers protocol (Jaffe assay method, Roche/Hitachi Cobas C system Roche diagnostics, Indianapolis, IN, USA). The plates were spectrophotometrically read at 490nm (with and without a creatinine quencher to determine the final absorbance values, which are the definitive urinary creatinine values of the sample (figure 5.5).

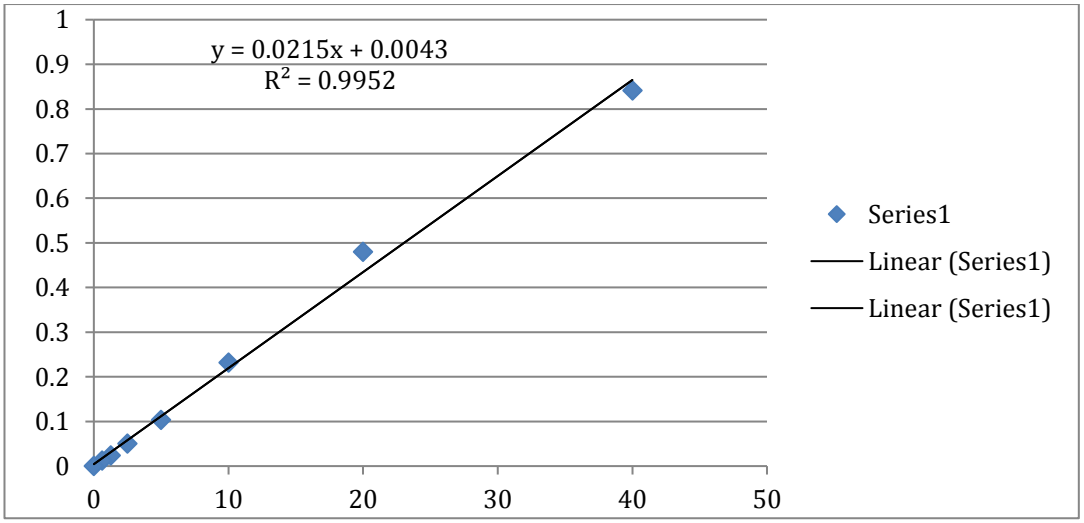


Figure 5.5: Urinary Creatinine assay standard curve

5.6.6 Estimation of urinary KIM-1/Creatinine

KIM-1 (pg/ml) corrected for urinary creatinine excretion (mg/gram) was expressed as KIM-1/Cr (ng/mg).

5.7 Withdrawal from the study

All study participants were free to withdraw from the study at any time. Following this, their samples were removed and destroyed in keeping with regulatory requirements.

5.8.0 Study outcomes

This study met the criteria outlined by the medical research council (MRC) guidelines for complex interventions. This is borne out of the impact incorporation of pharmacogenetic outcomes will potentially have on the overall determinants (including clinical and biochemical) of kidney injury in this cohorts of patients.

5.8.1 Primary outcome

- The primary outcome measure is elevated median of KIM-1/Cr at baseline and 24 weeks. I described DETIKI patient cohort with the view to validate the utility of LMWP as biomarkers for monitoring of renal function in HIV positive patients exposed to ART drugs.

5.8.2 Secondary outcomes

- Correlation between SNP's/haplotypes of genes encoding transport proteins involved with the bio-disposition of ART drugs, and LMWP.
- Correlation between KIM-1/Cr and other traditional markers of kidney injury (eGFR, Urine PCR, etc.)
- Prevalence of kidney injury in HIV positive patients on ART drugs

5.9 Safety consideration

The safety of ART drugs in HIV-positive patients has been widely described. As this is not an interventional trial, study subjects recruited to both arms underwent their usual point of care monitoring for concurrent ART drug treatment consistent with national and local guidelines.

5.10 Sample size estimation

There are few robust studies in literature to enable objective estimation of sample size, largely because this is a developing field. This study was conducted as a pilot,

therefore I estimate that assuming a coefficient of variation (CV) of about 5% in median KIM-1(159), a sample size of 200 patients (cases), and 100 controls for the cross sectional sub-study, and 75 for the prospective longitudinal sub-study will give us about 80% power to detect 20% difference in KIM-1 population mean.

5.11 Data management

All data collected in course of the study was recorded in an individualised subject specific case report form (CRF). In order to maintain confidentiality, only subject number and initials on the CRF were used to identify study subjects.

5.12 Ethics and study sponsorship

The study protocol, patient information, consent forms, available safety information, study participants recruitment procedures, information about payments, and compensation available to the subjects as well as documentation evidencing the Investigator's qualifications was submitted to the North Manchester research ethics committee for ethical review and approval according to local regulations, prior to commencement of the study. Following initial review of the study protocol, a provisional approval was granted with suggested changes to study documentation. An amendment to this effect was re-submitted to ethics committee, following which a final approval was granted for commencement of the study.

5.13.0 Results

5.13.1 Patient's demographics and clinical characteristics

Table 5.2 gives a summary of baseline characteristics of the study population (cross-sectional cohort). About 73.7%, and 81% of patients recruited into the cross sectional and prospective arms of the study respectively were Caucasians. This perhaps reflects the demography of the main enrolment site (Royal Liverpool University

Hospital, the United Kingdom). Amongst 114 cross-sectional patient cohorts, mean age was 43.1 years (SD ±9.9), with about 70.2% male population. Proteinuria (defined as urine PCR ≥20mg/mmol) was present in about 20.9% of cross sectional patient cohort with a higher proportion in older patients compared with their gender-matched cohorts (49.3mg/mmol vs. 41.4mg/mmol, respectively $P = 0.002$). Median TDF exposure was 36 months (IQR 22.5, 72). KIM-1/Cr had a non-parametric distribution as shown in figure 5.6. Median KIM-1/Cr was 4.17ng/mg (IQR 1.5, 8.2) with an upper quartile threshold of ≥8.6ng/mg.

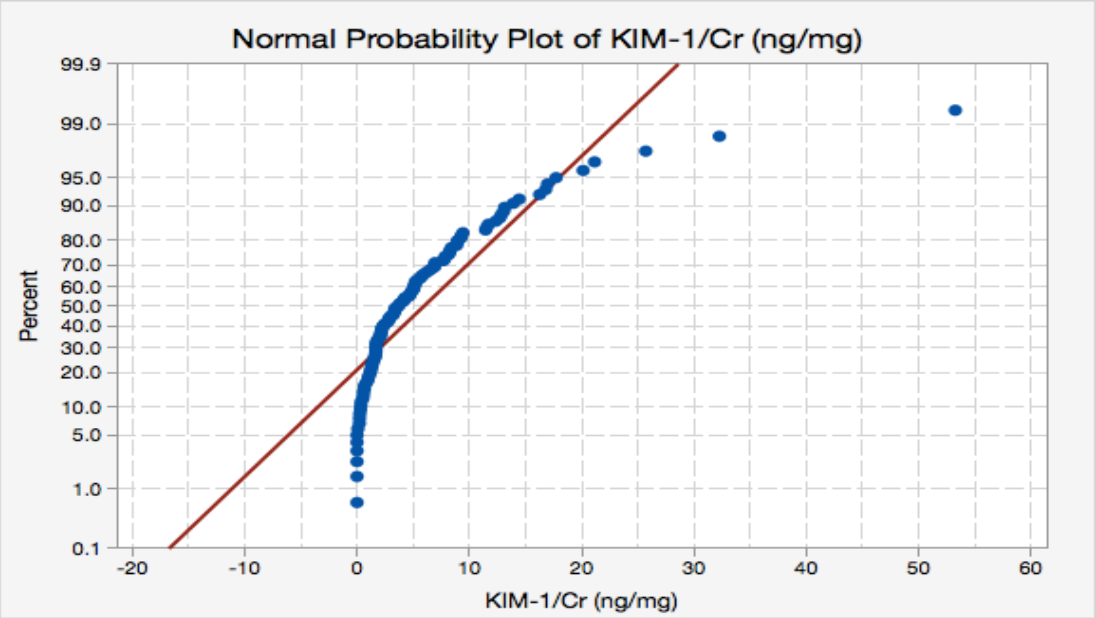


Figure 5.6: Probability plot of the distribution of KIM-1/Cr amongst DETIKI cross sectional patient cohorts.

Table 5.2: Demographic and clinical characteristics of DETIKI cross-sectional cohort (*N* = 114)

Variable			***Number available for analyses (N)***
Age (years)	*Mean (SD)*	43.1 (9.9)	114
Male Gender	*N (%)*	80 (70.2)	114
White Caucasian	*N (%)*	84 (73.7)	114
Duration of HIV in years	*Med (IQR*)	8 (4, 12)	113
KIM-1/Cr (ng/mg)	*Med (IQR)*	3.7 (1.5, 8.2)	114
Serum Creatinine (mmol/l)	*Med (IQR)*	83.5 (69, 93)	114
eGFR (mL/min/1.73 m^2)	*Med (IQR)*	85 (74, 90)	114
Urine PCR (mg/mmol)	*Med (IQR)*	11 (8, 17)	68
Serum Phosphate (mmol/l)	*Med (IQR)*	0.96 (0.86, 1.06)	112
Total Cholesterol (mmol/l)	*Med (IQR)*	5 (4.5, 5.7)	110
HDL Cholesterol (mmol/l)	*Med (IQR)*	1.3 (1.1, 1.7)	108
Triglycerides (mmol/l)	*Med (IQR)*	1.2 (0.9, 1.9)	109
TDF exposure (months)	*Med (IQR)*	36 (22.5, 72)	54
Protease Inhibitor (PI) exposure	*N (%)*	45 (39.5)	105
Abacavir exposure	*N (%)*	19 (16.7)	114
CD4 count (cells/mm^3)	*Med (IQR)*	565 (401, 665)	101
Nadir CD4 count (cells/mm^3)	*Med (IQR)*	200 (95, 298)	99
Hepatitis B Positive	*N (%)*	23 (20.2)	96
Hepatitis C Positive	*N (%)*	5 (4.4)	109
Diabetes Mellitus	*N (%)*	4 (3.5)	106
Hypertension	*N (%)*	6 (5.3)	104
Smoking	*N (%)*	36 (31.6)	104

a. Standard deviation; b. number of patients; c. Median; d. Inter-quartile range

Table 5.3 gives the distribution of adjusted KIM-1/Cr amongst the various study cohorts. Patients with urine PCR ≥20mg/mmol had a higher median KIM-1/Cr (ng/mg) levels than their comparable controls (5.9ng/mg vs. 2.7ng/mg, confidence interval [CI] 2.4-4.8; *P* = 0.0001). About 5.3% (*N* = 6), and 3.5% (*N* = 3) of the study cohort were hypertensive or have incident Diabetes Mellitus respectively. The median nadir/current CD4 count were 200 cells/mm^3 (IQR 95, 298), and 565 cells/mm^3 (IQR 401, 665) respectively. The median eGFR was 85 mls/min/1.73m^2

(IQR 75.5, 90) with 5.3% of the cross sectional study cohort having chronic kidney disease stage 3 (CKD3, eGFR<60). Baseline eGFR levels were significantly lower in males than their age-matched female counterparts (82 vs. 90 mls/min/1.73m^{2,} respectively; CI 1.4-3.6; P = <0.0001). Table 5.4 gives comparative distribution of kidney surrogate biomarker levels amongst the various cross sectional patient cohorts.

Table 5.3: Distribution of various adjusted KIM-1/Cr * thresholds amongst the DETIKI Study populations

		Cross-sectional arm (on ART) *N* **= 104**	**Cross-Sectional arm (off ART)** *N* **= 10**	**Prospective arm** *N* **= 11**	**P**
	25th centile	1.67	1.93	3.27	0.66
	Median	4.14	4.69	4.01	**0.015**
	75th centile	8.63	5.3	4.89	0.46

* Urinary KIM-1 corrected for urinary creatinine excretion

5.13.2 Prospective sub-study

Amongst this longitudinal patient cohort (*N* = 11), mean age was 40 years (±7.3) with a median HIV duration of 3.5 years (IQR 1, 8). Median KIM-1/Cr (ng/mg) was 4.0 (IQR 3.6, 4.8). Median CD4 count (cell/mm^3), and HIV viral load (copies/ml) were 468 (453, 555), and 82 (27, 22700) respectively. All patients recruited to this

arm of the study had eGFR >60mls/min/1.73m^2 with a median urine PCR of 10mg/mmol (IQR 5, 14).

Table 5.4: Comparative distribution of kidney biomarkers amongst the cross-sectional patient cohorts (*N* = 114)

Variables		**All patients** *N* **=114**	**cross-sectional on ART** *N* **= 104**	**cross-sectional off ART** *N* **= 10**	**P**
KIM-1/Cr (ng/mg)	Med (IQR)	4.1 (1.7, 8.3)	4.15 (1.7, 8.6)	2.7 (1.9, 5.4)	0.1
Urine PCR[1] (mg/mmol)	Med (IQR)	11 (8, 17)	11 (8, 17)	9.5 (6, 122)	0.5
eGFR (mls/min/1.73m^2)	Med (IQR)	85 (75, 90)	85 (75, 90)	90 (63, 90)	0.2

1. Urine protein creatinine ratio

5.13.3 Urinary KIM-1/Cr concentration and ethnicity

Baseline KIM-1/Cr values were higher in patients of white Caucasian ethnicity (4.1ng/mg) compared with patients from other ethnic groups (1.1ng/mg, [CI 2.2-3.9], *P* = 0.005) figure 5.7. When stratified based on ART exposure, Caucasian patients either "on" or "off" ART drugs had a significantly higher baseline KIM-1/Cr (ng/mg) values compared to other ethnic cohorts (8.6 vs. 2.1, and 2.2 vs. 1.9 respectively, P = 0.008).

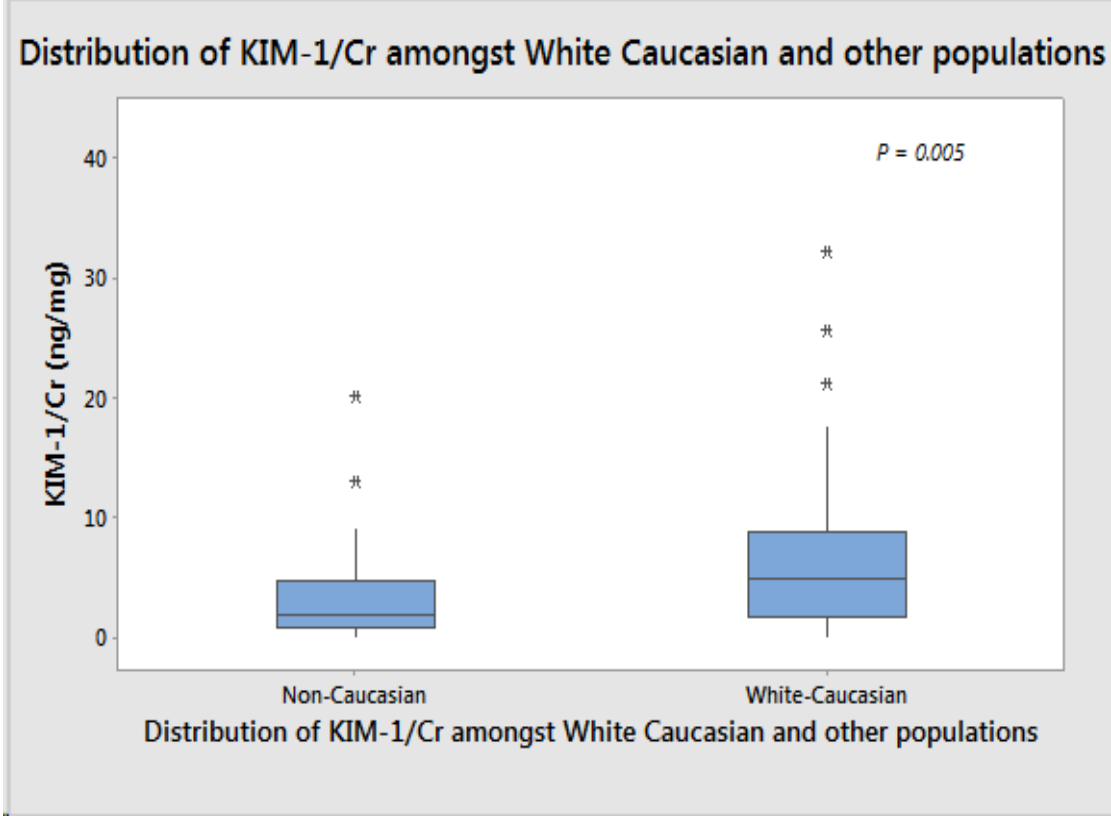


Figure 5.7: Distribution of KIM-1/Cr (ng/mg) amongst various ethnic cohorts. Irrespective of antiretroviral therapy drug exposure, patients of white Caucasian ethnicity maintained higher KIM-1/Cr levels compared to other ethnic cohorts

5.13.4 Antiretroviral drug exposure and kidney biomarker levels

Table 5.5 show detailed comparison of KIM-1/Cr as a function of ART exposure. KIM-1/Cr is uniformly distributed across all patient cohorts regardless of ART drug regimen. However, when stratified according TDF exposure, patients on TDF regimen had a significantly higher median KIM-1/Cr than comparable cohorts (figure 5.8).

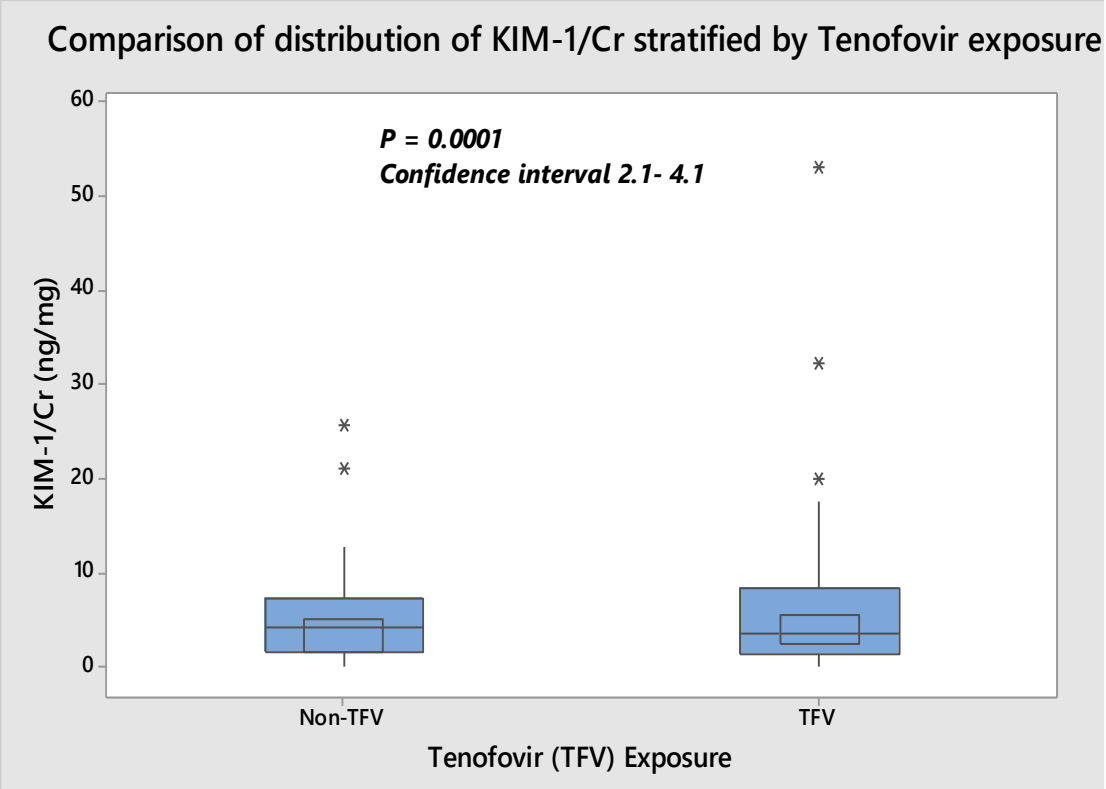


Figure 5.8: Box plot showing distribution of KIM-1/Cr (ng/mg) stratified by Tenofovir disoproxil fumarate exposure. HIV positive patients on TDF based regimen had significantly higher urinary KIM-1/Cr levels representing some degree of kidney impairment in this cohort of patients.

Patients on ART/PI regimen had higher median urine PCR compared to those on alternative regimen (P = 0.02). Amongst cross sectional cohorts, patients on PI based regimen had higher KIM-1/Cr (5.6 ng/mg) compared to their PI-naïve counterparts (3.4ng/mg) but this did not reached statistical significance.

5.13.5 Stratified protease inhibitor exposure and kidney biomarker levels

About 32.2% of patients within the cross sectional study cohort were exposed to boosted PI's (rtv boosted). The prevalence of ATV exposure was limited to about 9.6% of total PI exposure. See table 5.6 for summary of kidney biomarkers stratified according to various PI sub-classes.

Table 5.5: Comparative distribution of kidney biomarkers stratified by antiretroviral therapy regimen amongst DETIKI cross sectional cohort

	All patients *N* = **114**	**ART/TDF** *N* = **45**	**ART/PI** *N* = **11**	**TDF/PI** *N* = **31**	**Other cART** *N* = **18**	**P**
KIM-1/Cr (ng/mg) ***Med (IQR)***	3.7 (1.5, 8.2)	3.35 (1.6, 8.2)	5.37 (3.4, 10.3)	2.28 (0.48, 6.75)	6.17 (1.73, 9.0)	0.07
Urine PCR (mg/mmol) Med (IQR	11 (8, 17)	12 (9, 23.5)	15 (8.0, 67.2.)	8 (6, 11)	14 (8.2, 30)	**0.02**
eGFR (mls/min/1.73 m^2) ***Med (IQR)***	85 (74, 90)	82 (71, 90)	90, 77.5, 90)	82 (72.7, 90)	89 (64.5, 90)	0.78

Other ART: These include Integrase inhibitors, entry inhibitors, NNRTI's. ART: antiretroviral therapy; TDF: Tenofovir; PI: Protease inhibitors P value is for group comparison between ART classes

Table 5.6: KIM-1/Cr stratified by protease inhibitor (PI) exposure phenotypes

Variables	**Boosted PI'S (rtv)** ***N =37***	**Atazanavir based regimen** ***N = 11***	**P**
KIM-1/Cr (ng/mg) Median, IQR	5.4 (1.6, 9.1)	6.2 (3.2, 12.7)	0.23
Urine PCR (mg/mmol) Median, IQR	11 (6.5, 16)	11 (6, 12)	0.60
eGFR (mls/min/1.73m^2) Median, IQR	79 (72.5, 90)	89 (66, 90)	0.66
Serum creatinine (mmol/l) Median, IQR	82 (67.5, 94.5)	76 (67, 106)	0.93

5.13.6 Correlation between urinary KIM-1/Cr and traditional Markers of kidney injury

There were statistically significant correlations between urinary KIM-1/Cr with age, total CD4 count, and urine PCR (table 5.7). When stratified into quartiles, median KIM-1/Cr maintained strong linear positive correlation with urine PCR (r = 0.35, p = 0.003), table 5.8. It is noteworthy that urinary KIM-1/Cr (ng/mg) showed a positive correlation with urine PCR (a well-established marker of kidney injury in these cohorts of patients).

Table 5.7: Correlation matrix of various co-variates as a function of KIM-1/Creatinine (ng/mg)

		Age (Years)	**TDF exposure (months)**	**urine PCR**	**eGFR**	**Total CD4 (cells/ mm^3)**	**CD4 (Nadir)**
	Correlation Coefficient #	0.44**		0.20	-0.32**	0.11	-0.21*
	P	**0.000**		0.09	0.00	0.26	0.03
	Number available for analyses	113		67	113	101	99
	Correlation Coefficient	0.37**	-	0.34*	-0.36**	-0.15	-0.24
	P	0.006	-	0.03	0.00	0.28	0.08
	Number available for analyses	54	-	37	54	50	50
	Correlation Coefficient	0.300**	0.15	-0.02	-	0.06	-0.10
	P	0.001	0.27	0.86	-	0.54	0.28
	Number available for analyses	114	54	67	-	101	99
	Correlation Coefficient	0.23*	0.05	0.27*	-0.15	-.020*	-0.20
	P	0.01	0.68	0.03	0.12	0.04	0.05

**: Statistically significant correlations; #: Spearman's rho

The correlation matrix of urinary KIM-1/Cr as a function of other covariates is given in table 5.7. About 52% of this study population with eGFR greater than 60mls/min/1.73m^2 (and therefore normal kidney function) had KIM-1/Cr concentration higher the median threshold. This will suggest a degree of subclinical kidney injury in patients with hitherto normal eGFR (using a median KIM-1/Cr as a marker of kidney injury).

Table 5.8: Key correlation matrix between adjusted urinary KIM-1/Cr and other markers of kidney function

Variable	**Unadjusted KIM-1/Cr (ng/mg)**		**Median KIM-1/Cr (ng/mg)**		**Upper quartile KIM-1/Cr (ng/mg)**	
	R	*P*	R	*P*	R	*P*
Serum creatinine (mmol/l)	0.067	0.48	0.14	0.4	0.07	0.71
eGFR (mls/min/1.73m^2)	-0.14	0.13	-0.22	0.13	-0.19	0.3
Urine PCR (mg/mmol)	0.35	**0.003**	0.34	**0.06**	0.28	0.2

R = Spearman's correlation coefficient; significance level at <0.05

5.13.7 Diagnostic performance of KIM-1/Cr (ng/mg)

I generated AUC/ROC curves to evaluate the performance of various thresholds of KIM-1/Cr (ng/mg), serum creatinine (mmol/l), and eGFR (mls/min/1.73m^2) in differentiating patients with kidney injury from those with normal kidney function. Utilising urine PCR ≥20mg/mmol as cut-off for kidney impairment, AUC-ROC of KIM-1/Cr (ng/mg) was 0.68, (CI = 0.50-0.84, p = 0.04) figure 5.9.

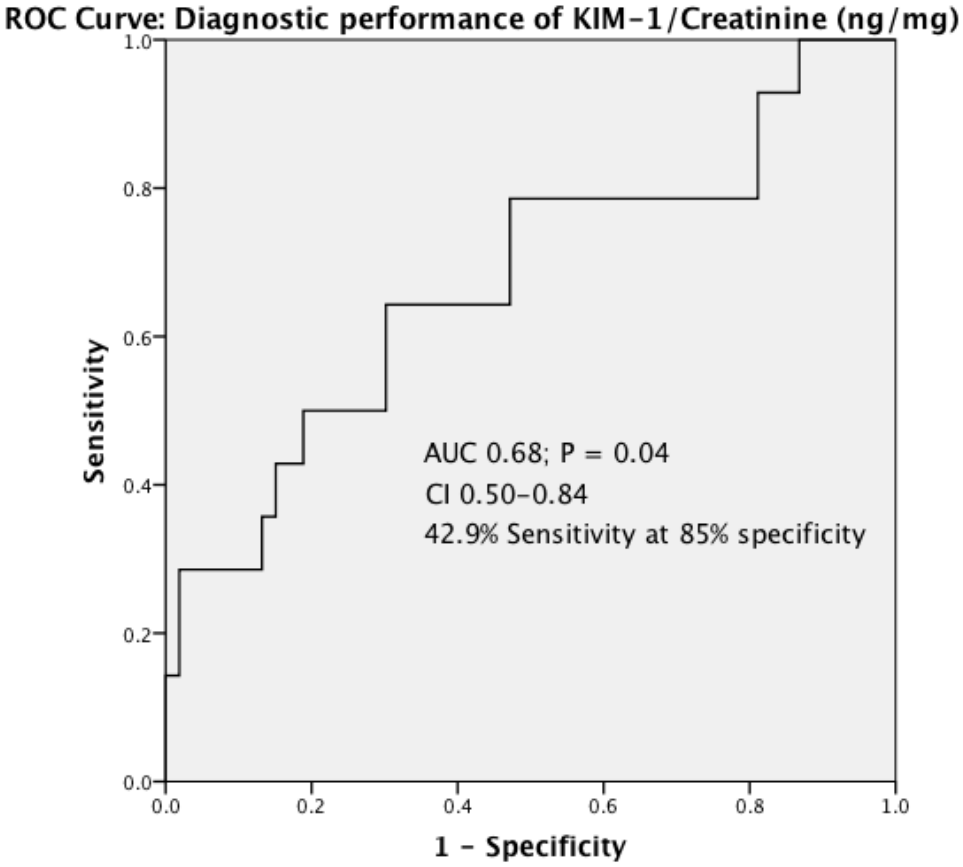


Figure 5.9: ROC analysis of KIM-1/Cr as a single variable. The area under the receiver operator curve is given (0.67) for prediction of kidney injury with 95% confidence interval. The higher and more leftward the KIM-1/Cr value, the greater its propensity to predict risk of kidney dysfunction

KIM-1/Cr (ng/mg) had a higher areas under the curve (AUC) compared with other established diagnostic markers for acute kidney injury in this population. The AUC's, as well as sensitivities and specificities of other biomarkers are depicted in Figures 5.10-5.11.

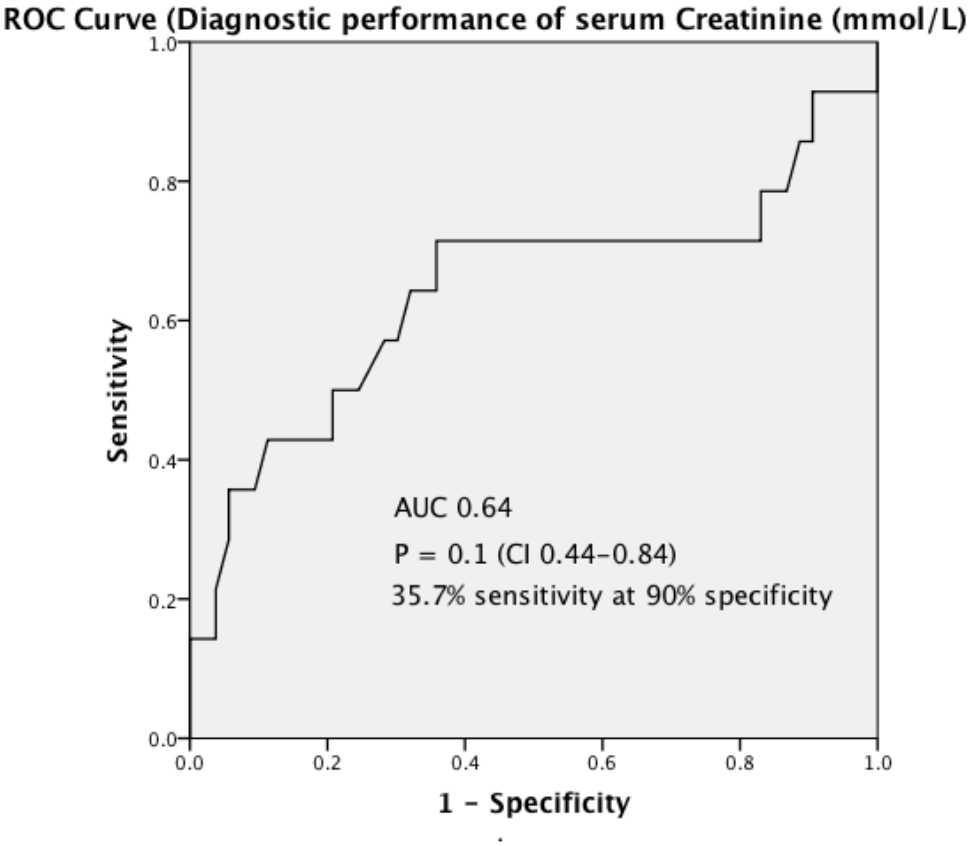


Figure 5.10: ROC analysis of serum creatinine (mmol/l) as a single variable. The higher and more leftward the serum creatinine value, the greater its ability to predict risk of kidney dysfunction

5.13.8 Predictors of kidney injury

I tested the diagnostic performance of median KIM-1/Cr (≥4.17ng/mg) as a predictor of kidney injury in HIV positive patients exposed to various ART drug regimen. Variables with P values <0.2 in univariate analyses (age, white Caucasian ethnicity,

TDF exposure (years), PI exposure, Abacavir exposure, eGFR, Urine PCR, Total and nadir CD4 count) were entered into multivariate analysis. By forward logistics regression (likelihood ratio), TDF exposure (per year increase) was significantly associated with risk of kidney injury in multivariate analyses (Odds ratio 1.4, CI 1.02-1.82, P = 0.034), Table 5.9.

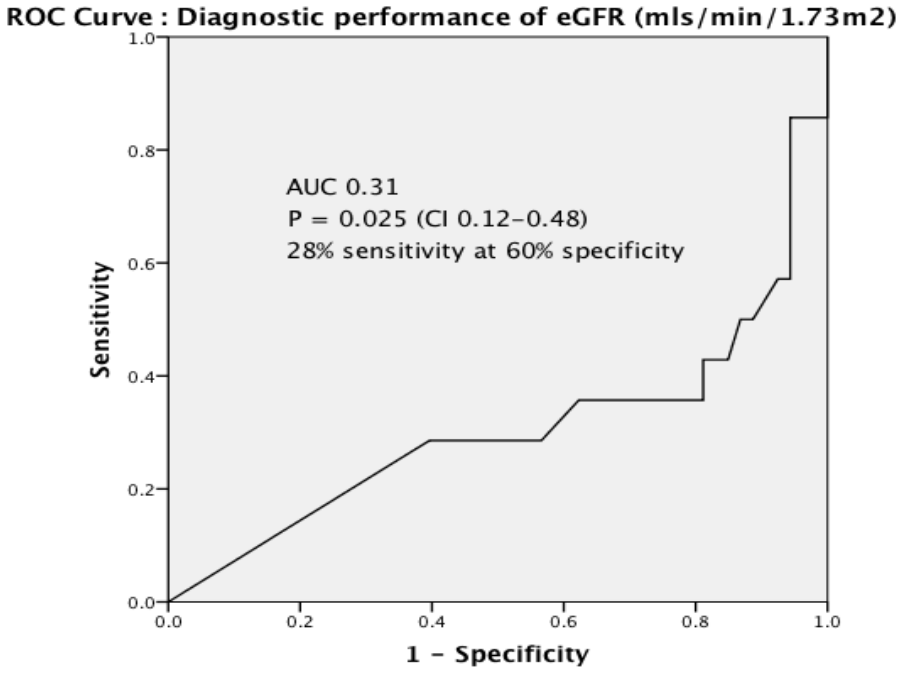


Figure 5.11: ROC analysis of e-GFR as a single variable. The area under the receiver operator curve is given for prediction of kidney injury with 95% confidence interval. The higher and more leftward the eGFR value, the greater its propensity to predict risk of kidney dysfunction

Table 5.9: Univariate, and multivariate adjusted odds ratios of median urinary KIM-1/Cr (ng/mg)

	Univariate		**Multivariate**	
	OR (CI)	**P**	**Adjusted OR (CI)**	**P**
Age (years)	1.1 (1.0-1.1)	**0.035**	--	--
Gender (male)	0.66 (0.28-1.5)	0.34	--	--
White Caucasian ethnicity	0.37 (0.14-0.95)	**0.04**	--	
HIV duration (per year increase)	1.1 (1.0-1.1)	**0.051**	--	--
Tenofovir disoproxil fumarate exposure (years)	1.2 (0.98-1.42)	0.08	**0.034**	1.4 (1.02-1.82)
HIV protease inhibitor exposure (months)	0.47 (0.21-1.1)	**0.074**		
Abacavir (ABC) naïve	4.4 (1.1-14.5)	**0.01**	--	--
Diabetes Mellitus	1.1 (1.3-7.6)	0.98	--	--
Hypertension (mmHg)	0.98 (0.98-5.1)	0.9	--	--
Smoking (Yes)	0.59 (0.25-1.3)	**0.22**	--	--
HIV RNA viral load (cells/mm^3)	1.0 (0.9-1.0)	0.7	--	--
eGFR (mls/min/1.73m^2)	0.97 (0.94-1.0)	**0.2**		
Urine PCR (mg/mmol)	1.0 (0.98-1.0)	**0.25**	--	--
Current CD4 count	0.9 (0.9-1.0)	**0.25**	--	--
Nadir CD4 count	1.0 (0.9-1.0)	**0.13**	--	--
Hepatitis C positive	0.98 (0.1-7.2)	0.9	--	--
Hepatitis B positive	0.5 (0.16-0.17)	0.3	--	--

5.14 Discussion

Traditional markers of kidney injury such as eGFR have increasingly been shown to detect kidney impairment later than tubular proteinuria in HIV positive patients exposed to ART drugs (161, 162). The increasing prevalence of ART related drug induced kidney morbidities meant that there is urgent need for novel surrogate markers that report sub-clinical level of kidney injury before routine kidney function tests (such as eGFR and urine ACR/PCR) become abnormal. Recently Low molecular weight proteinuria such as retinol binding protein (RBP) (162), KIM-1 (163, 164, 165), neutrophil gelatinase associated lipocalin (NGAL) (174) have been proposed as early markers of kidney injury in both HIV positive patients on ART and the general population (173), (174). In this cross sectional study, I have evaluated the association between traditional markers of kidney injury (such as eGFR, and urine PCR) in HIV positive patients on ART drugs, and a novel marker KIM-1/Cr (adjusted for urinary creatinine excretion) that has increasingly been shown to report kidney tubular injury early in the general population. I observed a higher median urinary KIM-1/Cr levels in these cohort of HIV positive patients (4.17ng/mg, IQR 1.68, 8.38) regardless of ART exposure compared with either healthy volunteers (0.097-0.39 ng/mg) (197), or patients with acute kidney injury in the general population (0.57925 ng/mg, IQR 0.3997-0.82972) (198). The median KIM-1/Cr for example in patients off ART in DETIKI study cohort was 4.6ng/mg. This may suggest a degree of sub-clinical kidney injury in this cohort of patients with normal kidney function (eGFR $\geq$60mls/min/1.73m^2). A number of reasons may account for this. The natural history of HIV related kidney involvement may be associated with up regulation of KIM-1 synthesis in kidney tubular cells with resultant increased excretion of KIM-1 independent of exogenous injury (including ART drugs). This is

supported by data from mechanistic studies that suggests tubular injury of diverse aetiologies resulting in up-regulation of this putative cell adhesion molecule (KIM-1) (163). In addition, previous reports have shown that both glomeruli, and distal convoluted tubules were early target sites for the HIV virus (199) further supporting the role of direct effect of the virus in up regulating KIM-1 expression. Whilst the exact role of the natural history of HIV in KIM-1 kinetics remains a matter of mechanistic debate, I hypothesise that it may (in common with other yet to be identified factors) account for its high baseline urinary excretion of KIM-1 in HIV positive patients either on or off ART drugs. Utilising median KIM-/Cr as a marker of kidney injury, I found TDF exposure to be a strong predictor of kidney dysfunction in HIV positive patients on ART. TDF and PI exposures (146, 147) have both been independently associated with increased risk of KTD from various mechanistic and systematic studies. Resultant TDF induced KTD is likely to result in either increased up regulation of KIM-1, or its increased kinetics and excretion in urine. Higher median KIM-Cr values in these cohorts of patients may reflect its potential role both as a marker and as predictor of adverse outcomes in patients on these treatment regimens.

I similarly found a high baseline KIM-1/Cr levels in patients of white Caucasian ethnicity compared to other cohorts. When stratified based on ART exposure, Caucasian patients maintained higher KIM-1/Cr values compared to other ethnic cohorts regardless of ART exposure. This may suggest an influence of ethnicity both in the expression (on kidney tubular cells), and possibly increased tubular excretion of KIM-1 from the kidneys. In the determination of reference urinary KIM-1 reference intervals in healthy children, McWilliams et al found a higher urinary KIM-1 concentration in individuals of Caucasian ethnicity compared to African-

American cohorts (200). Additionally, previous reports have highlighted potential ethnic bias in the expression and distribution of tubular proteinuria (201). In the women multi-agency study for example, Black ethnicity was associated with significant albuminuria as well as an independent risk factor for increased excretion of tubular biomarker proteins including interleukin-8 (IL-8), neutrophil gelatinase associated lipocalin (NGAL), α-1 microglobulin (A-1M) in HIV positive patients on ART (201). A probable explanation for this observation might be the role of genetics in influencing the adverse effects of ART on the kidneys, as well as expression and kinetics of these low molecular weight proteins. TDF induced kidney tubular dysfunction for example has a higher prevalence in white Caucasian males compared with other gender matched ethnic cohorts (107, 108, 109, 144, 149). Although association between TDF exposure and risk of KTD remains speculative, recently both mechanistic and pharmacogenetic studies have increasingly implicated the role of single nucleotide polymorphisms (SNP) of genes encoding proteins involved in the bio-disposition of TDF (107, 108, 110). It is probable that there might be an ethnic bias in the genes associated with synthesis and expression of KIM-1 on tubular cells. SNP of these genes therefore accounting for either increased or decreased expression of this putative glycoprotein on kidney tubular cells. The exact underlying mechanism for the increased urinary KIM-1 excretion in Caucasian population is still unknown, and will need exploration by further mechanistic work in this area.

I also observed a significant positive correlation between advancing age, white Caucasian ethnicity, and high urine PCR levels with KIM-1/Cr. In HIV-positive patients, higher urine PCR levels have always been associated with and utilised as a point-of-care marker of kidney dysfunction in these cohort of patients on ART (162).

The positive correlation reported by this study supports a potential role of KIM-1/Cr (a potential alternative marker of kidney dysfunction this cohort of patients). It is noteworthy that in this report urine KIM-1/Cr kinetics maintained an inverse relationship with eGFR. The latter is a well-established surrogate marker of kidney injury in both HIV positive cohorts and the general population, with lower levels associated with worsening kidney impairment. This will additionally support a possible role for urinary KIM-1/Cr as a potential marker of kidney injury in HIV positive patients exposed to ART drugs. In addition, I have shown that a significant number of patients in this study cohort with hitherto normal kidney function (eGFR (>60mls/min/1.73m^2) had median KIM-1/Cr (ng/mg) concentration significantly above diagnostic thresholds (for KIM-1) for kidney injury in the general population. This may suggest some degree of subclinical kidney injury that has been masked or not reported by kidney function as determined by the four variable eGFR. The novelty of this approach is the identification of these patients with significant diagnostic values of KIM-1/Cr (>4.17ng/mg) even when eGFR in these patients were reported as normal.

Serum creatinine and eGFR have remained the main diagnostic markers of acute kidney injury in both the general population and HIV positive patients. Despite this, KIM-1/Cr (ng/mg) had a comparatively higher significant *AUC* (0.68, P = 0.04, CI 0.5-0.84) compared to these established kidney markers in this study. This may additionally suggest a potential diagnostic role for KIM-/Cr in these cohorts of patients, and perhaps its ability to identify kidney injury earlier in these cohorts of patients than traditional markers of kidney injury. The higher proportion of proteinuria (urine PCR >20mg/mmol) in older cohort of patients in this study appears consistent with findings from the general population. Similarly, analyses of this study

cohort also showed a reciprocal relationship between eGFR and advancing age, another matrix that mirrors findings in the general population.

5.14.1 Study limitations

Admittedly, a number of limitations evident in this study including its relatively small sample size and cross-sectional design meant that causality could not be established with certainty based on its findings. This will require subsequent validation by large sample sized prospective studies. I will therefore advise caution in interpretation and application of these findings.

5.14.2 Conclusion

I have demonstrated that in HIV positive patients on ART, TDF exposure significantly predicts the risk of kidney injury as defined by median KIM-1/Cr (>4.17ng/mg). Additionally, HIV positive patients regardless of ART drug exposure had urinary KIM-1/Cr values well above diagnostic thresholds for either normal volunteers, or patients with AKI in the general population. This suggests a probable role for median KIM-1/Cr (>4.17ng/mg) as an early diagnostic biomarker in these cohorts of patients. Further exploitation/utility of this threshold (KIM-1/Cr >4.17ng/mg) at point for care (as a diagnostic marker of kidney injury) in these cohorts of patients will however need validation by a larger cohort of prospectively recruited patient population.

CHAPTER 6

FINAL DISCUSSION

6.1 Epidemiological perspectives of ART drug related adverse effects

Patient's socio-economic status have been shown to significantly impact on the reporting of ART drug related toxicities (68). This is in common with other factors such as study setting, as well as tools/algorithms adopted in the adjudication of adverse events (68). These factors wholly or in common account for marked variability in reported prevalence rates of ART drug related ADR's (68). Even with clear recommendation by different national and international ADR grading and reporting guidelines (70), this variability in prevalence rates have remained the same (70). The CONSORT statement (72), and its further extension (73) were aimed at improving the robustness and effectiveness of reporting of ADR's from systematic studies across a broad range of morbidities including HIV positive patients. What impact this has made to the reporting of ADR's is still unknown. But by far the Naranjo ADR probability scale represents the most robust and validated adverse events assessment tool currently in use both in clinical practice as well as research settings (71). Additionally, proven efficacy and safety in randomised controlled clinical trials have been employed as part of the benchmark for incorporation of various ART drugs into national and internationally sanctioned treatment guidelines (18). Therefore, the phenotype of drug related adverse effects reported from these studies and the populations they study do contribute significantly to the totality of the adverse events morbidity associated with these drugs. Nevertheless, post-marketing surveys (phase IV) captures other components of adverse effects morbidity not reported or limited by the relatively short observational period of systematic studies. I have explored this in Chapter three of this thesis with a descriptive analysis of the MHRA database.

Despite these efforts, the observation that industry funded RCT's tended to report almost all spectrums of ADRs (mild, moderate or severe), in contrast to non-profit studies that have emphasized on reporting serious adverse events does suggests some outstanding lopsidedness in the reportage of overall ADR epidemiological burden (69).

Amongst the various ART drug related clinical toxicity syndromes thus far described, TDF related kidney injury is increasingly becoming a subject of clinical concern over the past few years. In this thesis, I have examined the prevalence, pattern, as well as genetic, and non-genetic determinants of TDF induced kidney injury in these cohorts of patients.

6.1.1 Tenofovir induced kidney tubular dysfunction (KTD)

Despites its well-established efficacy and safety (144), recently TDF have been a subject of increasing concerns regarding its propensity to cause kidney related adverse effects (145). In a recent EUROSIDA report for example, a 10-year exposure to TDF was associated with about 16% risk of decline in eGFR (146). The magnitude of eGFR decline in this report was highest in patients on ATV/r regimen (22%), compared to other PI based regimen (8% and 11% for LPV/r and Indinavir respectively) (146). Patients on ART regimen containing both PI/TDF had a faster rate of decline (41%) in kidney function in the study (146). Despite these reports, the exact clinical phenotype, diagnostic markers, and mechanism of TDF related kidney injury remains a matter of unresolved mechanistic and epidemiological debate. A number of recent reports have suggested kidney tubular dysfunction (KTD) and its extreme form (Fanconi syndrome) as the predominant clinical and laboratory phenotypes of TDF related kidney injury (144, 145). The reported prevalence of clinically significant TDF related kidney injury is variable, but it has been estimated

to range between 2-11% (147, 148, 149). The long-term effects of TDF on kidney function however still remain unknown. Analyses of the five and ten year Gilead follow-up studies (GS934, and GS903E respectively) have shown that long-term eGFR stabilises despite initial decline (202, 203, 204, 205).

6.2.0 The Role of *ABCC2* and *ABCC10* genetic polymorphisms, and risk of kidney tubular dysfunction following exposure to TDF: A systematic review and Metanalysis

Single nucleotide polymorphisms (SNPs) of genes encoding transport proteins involved in the bio-disposition of TDF have been examined in recent studies, especially their potential association with increasing risk of kidney injury in these cohorts of patients (107, 108, 110, 115). The discordant outcomes reported by these various studies meant that uncertainty remains as to the exact role of these SNP's in the pathogenesis of TDF related kidney toxicity. Most notably are SNP of genes encoding transport proteins such as *ABCC2 24CC* (MRP2*),* and *ABCC10* (MRP7) in HIV positive patients on ART. In this report, we have evaluated current evidence by carrying out a systematic review, and metanalysis of studies examining the role of *ABCC2* (MRP2), *ABCC4* (MRP4), *ABCC10* (MRP7), and *ABCB1* (*p*-glycoprotein) SNP's and risk of TDF induced KTD in HIV positive patients on ART. This is with the view to establish if an association exist, as well as its magnitude and potential utility in triage of these cohorts of patients before commencement of treatment.

We found significant association between possession of the *ABCC10* SNP (rs9349256), and risk of TDF induced KTD in HIV positive patients on ART. This is consistent with the seminal report by Pushpakom et al (110) who first established MRP7 as a putative TDF transporter, and subsequently confirmed association between SNP's of its gene (*ABCC10*, rs9349256, rs2125739) and risk of TDF

induced KTD. Conversely, we found no significant association between the multiply studied *ABCC2 24CC* genotype (rs717620, MRP2) and risk of KTD. This is hardly surprising, because as was exhaustively expounded in other sections of this thesis, MRP2 (encoded by *ABCC2*) have not been shown to be a putative TDF transporter. Additionally, whilst initial reports such Rodriquez-Novoa et al (107) have suggested its association with increased risk of KTD, subsequent reports (115) have failed to demonstrate any significant association. It is probable that the various modes of adjudication of kidney function (eGFR, diagnostic criteria for Fanconi syndrome etc.) adopted by the studies included in the review, as well as their small sample size may have accounted for the discrepancy of these reports.

6.2.1 Strengths of the study

This review represents the first attempt at comprehensive evaluation of current evidence of association between SNP's of genes encoding TDF transport proteins and risk of kidney injury in HIV positive patients. Despite its relatively small size (borne out of small sample size of constituent studies), it has provided a signal potentially invaluable in the design of future prospective pharmacogenetic studies especially as it pertains to the role of the intronic *ABCC10* (MRP7, rs9349256) SNP.

6.2.2 Limitations

A major imitation of this study is the relatively small size of its constituent studies. Additionally the lack of agreement in the definition of what constitutes kidney tubular dysfunction in the constituent studies may have confounded the true homogeneity/heterogeneity of our pooled data, and by implication reported association. Consequently, it is difficult to definitely establish causality between possessions of these SNP's and risk of KTD following TDF exposure with certainty. Despite this however, it provides the first attempt at comprehensive assessment of

current evidence, which will be invaluable in the design of future prospective pharmacogenetic studies.

6.2.3 Conclusion and future perspectives

The intronic *ABCC10* (MRP7, rs9349256) SNP was consistently associated with increased of TDF induced KTD in HIV positive patients on ART.

6.3.0 The pattern and phenotype of TDF induced kidney injury

Since the first reported association between TDF exposure and the risk of kidney injury, uncertainty remains as to its true pattern as well as clinical/laboratory phenotypes that define it (107, 144, 149). Without a thorough understanding of these two key variables (pattern of injury and laboratory/clinical phenotype), it becomes impossible to accurately identify patients at risk and therefore reduce further harm by opting for an alternative ART regimen and or ensuring more robust clinical/laboratory monitoring. Most of the initial reports in this area derive from trial cohorts of HIV positive patients exposed to TDF (145, 146). How much of the hospital observational kidney toxicity phenotype mirrors "real-life" experience of TDF in these cohorts of patients outside of clinical trial environment remains a matter of epidemiological debate (144). This study represents the first attempt at exploration of an observational database (the MHRA yellow card records) to ascertain the pattern of kidney injury in HIV-positive persons exposed to TDF. Over a 9-year observational period, I identified about 106 patients who satisfied criteria for the definition of acute kidney injury (33%), Fanconi syndrome (17%), and kidney tubular dysfunction (50%). This was out of a total of 407 yellow cards reviewed of patients who developed suspected ADR on TDF and had yellow cards submitted to MHRA. The higher percentage of KTD representing the milder form of TDF related kidney injury in this study population is in agreement with what has thus far been

reported from previous studies (107, 108, 110). The median duration of TDF exposure was 316 (IQR 120,740) days, with those having the extreme kidney toxicity phenotype (Fanconi syndrome) having the highest duration of exposure to TDF of (419 days, IQR 246, 1000). However, the relationship between duration of TDF exposure, and the development of the most extreme phenotype of TDF induced KTD is still uncertain.

I found a higher number of reported TDF related ADRs from 2005 to 2006, and then followed by a gradual decline thereafter. This perhaps reflects initial periods of increasing awareness of TDF related ADR's, resulting in increased monitoring, and perhaps a probable subsequent clinical caution with the use of TDF. I could not accurately estimate the impact of protease inhibitor (PI) exposure on the pattern of TDF-related injury in this cohort because of missing data on PI exposure in the study population. About four patients out of the entire study cohort had data on PI exposure. All patients on boosted Atazanavir (n = 2) had KTD, whilst those on boosted Lopinavir and Indinavir had Fanconi syndrome and KTD respectively. Despite this relative paucity of PI-exposure data, association of all PI's in this study cohort with varying degrees of KTD (including Fanconi syndrome) perhaps reflects and agrees with the totality of evidence thus far reported from most observational and clinical trial cohorts (154, 153, 171, 191). The role of PI exposure in the pathogenesis of TDF-related injury has continued to be a subject of intense mechanistic and therapeutic debate (144). In the recent analyses of the Swiss HIV study cohort database, contemporaneous administration of TDF with boosted Atazanavir or Lopinavir was associated with a greater initial rate of decline in e-GFR compared to TDF/EFV regimen (189). Additionally, Cao et al (190) recently reported a greater rate of decline in kidney function in a cohort of Chinese patients

on TDF+PI/r based ART regimen compared to those on non-TDF regimen at 48 weeks. Amongst suggested mechanisms for the role of PIs in the TDF-related kidney injury includes but not limited to PI-mediated increase in plasma concentration of TDF (191). Patients classified as Fanconi syndrome had the highest frequency of hospitalization (55.6%) compared to other patient's cohorts. As alluded to in earlier sections of this thesis, Fanconi syndrome represents the most extreme phenotype of TDF related kidney toxicity, and will probably explain its association with the highest morbidity seen in this cohort. Additionally, I also found complete restoration of kidney function in about 50% of this study cohort. This mirrors what has been reported by a number of systematic studies exploring TDF related kidney morbidity. I also found all reported deaths in this study cohort to be due other HIV related complications and not progressive kidney disease or TDF exposure.

6.3.1 Strengths of the study

This study represent the first attempt at interrogation of such a observational data scheme in order to address the uncertainty regarding the pattern of TDF related kidney injury. Having an observational cohort perspective to what has thus far been established from RCT's will prove invaluable in the design of future prospective studies aimed at exploring other outstanding clinical/mechanistic themes in regards to TDF related kidney injury.

6.3.2 Study limitations

This study is limited by previously identified impediments encountered in the course of exploring such observational data schemes. Its descriptive design for example, as well as difficulties with accurately estimating the prevalence of TDF related kidney injury suggests caution in the application of this study outcomes and limits any ability to estimate true incidence of TDF-related kidney injury. Additionally, issues

of under reporting of yellow card records suggest caution in establishing definite causality between TDF exposure and risk of kidney injury in these cohorts of patients. Furthermore, been a developing field, the absence of a universally accepted validated definition of what constitutes tubular dysfunction in HIV-positive patients on TDF makes objective adjudication of the Yellow Card reports difficult. Despite this however, this study outcome fits within the framework and narrative of the phenotype of TDF related KTD thus far expounded by systematic studies.

6.3.3 Future perspectives

Whilst this study may have provided an epidemiological perspective of the pattern of this evolving morbidity, however in order to accurately characterise the exact pattern and phenotype of TDF induced KTD, there will be need for further studies designed to explore other observational databases with the view to compliment and support data from systematic studies.

6.4.0 Association between *ABCC2* and *ABCC10* single nucleotide polymorphisms, and risk of kidney injury following Tenofovir Disoproxil Fumarate (TDF) exposure: A candidate gene study

This study represents the first attempt at exploring the association between thresholds of novel kidney markers such retinol binding protein (corrected for urinary creatinine excretion [RBPCR >17μg/mmol]), and SNP's encoding transport proteins involved in the bio-disposition of TDF (such as *ABCC2*, *ABCC4*, *ABCC10, OAT1, and OAT3*) in these cohorts of patients. I found possession of genotype *CC* at position 24 of the *ABCC2* gene to confer protection against risk of KTD in HIV positive patients exposed to TDF. This study is the first report suggesting a protective role for possession of this SNP's in HIV positive patients on TDF and therefore at risk of KTD. Previous reports have either reported increased risk of KTD with this SNP, or

have shown no effect depending on the method of adjudication of kidney function (107, 108, 109, 115). Most notably, studies utilising eGFR as marker of kidney dysfunction have shown no association between possession of the *ABCC2 24CC* genotype and risk of KTD in HIV positive patients on TDF-based ART regimen. This is probably expected given that tubular dysfunction rather than glomerular injury has been suggested as the underlying pathogenetic mechanism for TDF related kidney injury. The evident discordance in the outcomes of these studies (107, 108, 110, 115, 116) may be due to a number of factors; firstly, differences in outcomes measures (e.g. eGFR, LMWP, urine PCR etc.) utilised to evaluate kidney function may have accounted for either under, or over reporting of kidney impairment. Certainly, from most reports, eGFR tends to under report early stages of TDF related kidney impairment (162). Secondly, all of the pharmacogenetic reports thus far exploring SNP's of genes encoding TDF transport proteins and risk of KTD have been relatively small sample sized studies significantly underpowered to robustly report any definitive causality between possession of *ABCC2 24CC* genotype and risk of KTD. Additionally, I found no significant association between other evaluated SNP's such as *ABCC4*, *OAT1*, and *OAT3,* and the risk of TDF induced KTD.

6.4.1 Strengths of the study

Despite its novelty, this study represent the first attempt at exploring the relationship between possession of *ABCC2 24 CC* genotype, and risk of KTD in HIV positive patients exposed to TDF (by utilising a RBPCR as a marker of kidney injury). What has hindered utility of previous surrogate markers such as the previously reported diagnostic criteria of KTD and Fanconi syndrome (107) is their impracticability as point of care assays. Conversely, LMWP (RBP, KIM-1, NGAL, etc.) are increasingly been shown to be potential markers of KTD supported by their

additional ease of point of care assay. Nonetheless, urine ACR/PCR and eGFR for now remains the standard laboratory makers of kidney dysfunction in HIV positive patients or ART until validation of these LMWP by future prospective studies

6.4.2 Limitations of the study

This study is limited partly by its relatively small sample size, which makes robust association of causality difficult. Secondly, it's utility of a surrogate marker of kidney dysfunction (RBPCR) still undergoing evaluation as a point of care assay, and extraction of DNA from serum (rather than whole blood) suggests caution in the interpretation of these findings. Nevertheless, these findings are consistent with some of the earlier pharmacogenetic reports in this area and may not have been significantly confounded by these limitations.

6.4.3 Future perspectives

A large prospective pharmacogenetic study encompassing various ethnic populations and utilising currently validated markers of kidney dysfunction in these cohorts of patients is required to robustly and conclusively address the remaining uncertainty regarding the role of genetics in the pathogenesis of TDF induced KTD. Such study will be enhanced by incorporation of key pharmacogenetic questions proposed by Jorgenson et al (182) in its design to avoid the pit falls that has been the theme of previous pharmacogenetic reports in this area.

6.5.0 Association between KIM-1/Creatinine and risk of kidney dysfunction following exposure to ART drugs

The limitations of traditional markers of kidney injury such as eGFR and urine ACR/PCR in the diagnosis of ART-related kidney dysfunction are well established (162). This is more so with TDF induced kidney injury, which has been characterised

as tubular, rather than glomerular injury. This study has explored the correlation and probable diagnostic utility of KIM-1/Cr in HIV positive patients "on" and "off" various ART regimen. Previous studies in HIV population have explored relationship between thresholds of KIM-1, and kidney dysfunction/survival in pregnant women (201). Adopting a separate cross-sectional and prospective design, I explored the relationship (including potential diagnostic utility of this biomarker) in a cohort of HIV positive patients with varied morbidities and on various ART regimen. I found a higher baseline median (≥4.17ng/mg urinary KIM-1/Cr levels in HIV positive patients both "on" and "off" ART drugs compared to either non-HIV positive normal volunteers (0.39 ng/mg) (197), or those with acute kidney injury in the general population (0.57 ng/mg) (198). This may suggest a degree of sub-clinical kidney injury in this cohort of HIV positive patients with "normal" functioning kidneys (eGFR ≥60mls/min/1.73m^2). It is still unclear why these cohorts of patients have a higher baseline KIM-1/Cr concentration. HIV/AIDS associated kidney morbidities including possibly HIV associated nephropathy (HIVAN) might be associated with up regulation of KIM-1 synthesis in kidney tubular cells with resultant increased excretion of KIM-1 independent of exogenous injury (including ART drugs). This is supported by data from mechanistic studies, which showed that tubular injury of diverse aetiologies results in up-regulation of this putative cell adhesion molecule (KIM-1) (163). In addition, previous reports have shown that both glomeruli, and distal convoluted kidney tubules were early target sites for the HIV virus (199), further supporting the role of direct effect of the virus in up regulating KIM-1 expression. Whilst the exact role of the natural history of HIV/AIDS in KIM-1 kinetics remains a matter of mechanistic debate, I hypothesised that it may (in common with other yet to be identified factors) account for its high baseline urinary

excretion in HIV positive patients either “on” or “off” ART drugs. Mechanistically, it is important to assess KIM-1 kinetics different from changes in eGFR as the latter has been suggested to report predominantly glomerular injury. Conversely, kidney tubular injury has been the hallmark of a number of ART drugs, most notably TDF. Additionally, I found a statistically significant correlation between urinary KIM-1/Cr with age, white Caucasian ethnicity, and urine PCR. Urine PCR is a well-established marker of kidney injury in both HIV positive patient cohorts as well as the general population. Its significant positive correlation with a threshold of KIM-1/Cr may suggest a probable diagnostic role for this biomarker in these cohorts of patients. I have already shown significant urinary levels of KIM-1/Cr in patients with normal eGFR. About 52% of this study population with “normal” kidney function (eGFR greater than 60ml/min/1.73m^2) have KIM-1/Cr values higher the median threshold (reported by manufacturers or the general population). This will suggest a degree of subclinical kidney injury in patients with hitherto normal eGFR. I further assessed agreement between KIM-1/Cr, and a current diagnostic benchmark of TDF induced kidney injury (urine PCR >20mmol/L) by ROC analysis. Utilising urine PCR>20mg/mmol as a marker of kidney injury, I found KIM-1/Cr (ng/mg) having a higher AUC 0.67 compared to either serum creatinine (0.64), or eGFR (0.31) in diagnosing patients with kidney injury.

6.5.1 Strength of the study

This study is amongst the first to explore the utility of novel LMWP such as KIM-1/Cr as potential surrogate markers of kidney dysfunction in a population of HIV positive patients on ART, whose pattern of kidney toxicity appears to be under diagnosed by other more established traditional markers of kidney dysfunction. This study established significant correlation between a novel marker of kidney

dysfunction (KIM-1/Cr) with other traditional markers of kidney injury such as urine PCR.

6.5.2 Limitations of the study

This study is limited by its relatively small sample size, and cross-sectional design. This meant that causality could not be established with certainty based on its findings. A further validation of these findings will be required by prospective studies adequately powered to explore these findings. I will therefore advise caution in interpretation and application of these findings.

6.5.3 Future perspectives

Whilst this study may have provided an epidemiological perspective of the pattern of this evolving morbidity, however in order to accurately characterise the exact pattern and phenotype of TDF induced KTD, there will be need for further studies designed to explore other observational databases with the view to compliment and support data from systematic studies.

A large prospective pharmacogenetic study encompassing various ethnic populations and utilising currently validated markers of kidney dysfunction in these cohorts of patients is required to robustly and conclusively address the remaining uncertainty regarding the role of genetics in the pathogenesis of TDF induced KTD. Such study will be enhanced by incorporation of key pharmacogenetic questions proposed by Jorgenson et al (182) in its design to avoid the pit falls that has been the theme of previous pharmacogenetic reports in this area. For now, traditional markers of kidney function such as eGFR, urine PCR/ACR continue to serve as the point-of-care assays for the determination of kidney injury in all cohorts of HIV positive whether “on” or “off” ART drugs.

Additionally, adequately powered cross sectional and prospective studies are needed to robustly address both association, and agreement between novel kidney biomarkers such as KIM-1, and RBPCR with other traditional markers of kidney dysfunction. This is important as LMWP (such as KIM-1 and RBPCR) are increasingly evolving as early diagnostic markers of kidney injury in the general population. Their utility in ART drug related kidney injury cohort would be invaluable to the early detection and management of this rising morbidity. Having robust data attesting to their agreement with traditional markers (such as eGFR and urine PCR) will no doubt enhance their reliability, and acceptance as point of care assays for monitoring of HIV positive patients on ART drugs.

7.0 References cited

1. Gao F, Bailes E. Origin of HIV-1 in the chimpanzee Pan troglodytes. Nature. 1999;6718:436-41.
2. Cantani A. New human and simian HIV-related retroviruses (HIV-2, HTLV-IV, SIV)". Riv Eur Sci Med Farmaco. 1988;10(6):467-72.
3. World Health Organisation (WHO) global health observatory data:URL: http://www.who.int/gho/hiv/en/. Accessed on 23/072015.
4. Aghaizu A, Brown AE, Nardone A, Gill ON, Delpech VC, & Contributors. HIV in the United Kingdom 2013 Report: data to end of 2012. Public Health England, London. 2013.
5. HIV and AIDS reporting section: Centre for Infectious Disease Surveillance and Control (CIDSC), Public Health England. Accessed on 15/02/2015. 2015.
6. Bentwich Z, Kalinkovich A. Immune activation is a dominant factor in the pathogenesis of African AIDS " Immunology Today. Immunology Today. 1995;16(4):187-91.
7. UNAIDS . Report on the global AIDS epidemic2015.
8. Berger E, Doms R, Fenyö E, Korber B, Littman D, Moore J. A new classification for HIV-1. Nature. 1998;391(391):240.
9. Reeves JD, Doms RW. Human immunodeficiency virus type 2. Journal of General Virology. 2002;83(6):1253-65.
10. Kwong PD. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998;393:648-59.
11. Coakley E, Petropoulos C, Whitcomb J. Assessing chemokine co-receptor usage in HIV. Current Opinion in Infectious Diseases. 2005;18(1):9-15.
12. Yu SF, Baldwin DN, Gwynn SR, Yendapalli S, Linial ML. Human foamy virus replication: a pathway distinct from that of retroviruses and hepadna viruses. Science.1996;271:1579-82.
13. Fassati A, Goff SP. Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. Journal of Virology. 2001;75:3626-35.
14. Engelman A, Mizuuchi K, Craigie R. HIV-1 DNA integration: Mechanism of viral DNA cleavage and DNA strand transfer. Cell.1991;67:1211-21.

15. Brik A, Wong CH. HIV-1 protease: mechanism and drug discovery. Organic Biomolecular Chemistry. 2003;1(1):5-14.
16. Engelman A, Cherepanov P. The structural biology of HIV- mechanistic and therapeutic insights. Nature Reviews Microbiology. 2012;10:279-90.
17. May MT, Vehreschild JJ, Gill MJ, Obel N, Crane HM, et al on behalf of the Antiretroviral Therapy Cohort collaboration. Survival of HIV positive patients starting antiretroviral therapy between 1996 and 2013: a collaborative analysis of cohort studies. The Lancet HIV. 2017;4(8):349-56.
18. Churchill D, Waters L, Ahmed N, Angus B, Boffito M, Bower M, et al. British guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy. HIV Medicine. 2016;17:2-104.
19. Mitsuya H, Broder S. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 20, 30 dideoxynucleosides. Proceedings of National Academy of Science. 1986;83:1911-5.
20. Cheng YC, Dutschman GE, Bastow KF, Sarngadharan MG, Ting RY. Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. Journal of Molecular Biology. 1987;262:2187-9.
21. Balzarini J, Herdewijn P, De Clercq Eea. Differential patterns of intracellular metabolism of 2, 3-didehydro-2, 3-dideoxythymidine and 3-azido-2, 3-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. Journal of Biological Chemistry. 1989;264:6127-33.
22. Brinkman K, Kakuda TN. Mitochondrial toxicity of nucleoside analogue reverse transcriptase inhibitors: a looming obstacle for long-term antiretroviral therapy? Current Opinion in Infectious Diseases. 2000;13:5-11.
23. Anderson PL, Kakuda TN. The cellular pharmacology of nucleoside transcriptase inhibitors and its relationship to clinical toxicities. Clinical Infectious Diseases. 2004;38:743-53.
24. Hien H, Meda N, Diagbouga Sea. 24-Month adherence, tolerance and efficacy of once-a-day antiretroviral therapy with didanosine, lamivudine, and efavirenz in African HIV-1 infected children: ANRS 12103/12167. African Health Sciences. 2013;13(2):287-94.

25. Longhurst HJ, Pinching AJ. Drug points: pancreatitis associated with hydroxyurea in combination with didanosine. British Medical Journal 2001;322(7278):81.
26. Aboulafia DM. Acute pancreatitis: a fatal complication of AIDS therapy. Journal of Clinical Gastroenterology. 1997;25(4):640-5.
27. Barmada MM, Pfützer RH. SPINK1/PSTI polymorphisms act as disease modifiers in familial and idiopathic chronic pancreatitis. Gastroenterology. 2000;119:615-23.
28. Felley C, Morris M. The role of CFTR and SPINK-1 mutations in pancreatitis disorders in HIV positive patients: a case-control study. AIDS. 2004;18 :1521-7.
29. Reisler RB, Murphy RL, Redfield RR, Parker RA. Incidence of pancreatitis in HIV-1-infected individuals enrolled in 20 adult AIDS clinical trials group studies: lessons learned. Journal of acquired immune deficiency syndrome. 2005;39(2):159-66.
30. Department of Health and Human Services 2013.
31. Ivetac A, McCammon JA. Elucidating the Inhibition Mechanism of HIV-1 Non-Nucleoside Reverse Mechanism of Inhibitors through Multicopy Molecular Dynamics Simulations. Journal of Molecular Biology. 2008;388(3):644-58.
32. MP dB. Nonnucleoside reverse transcriptase inhibitors NNRTIs their discovery development and use in the treatment of HIV1 infection A review of the last 20 years (1989-2009). Antiviral Research 2009;85(1):75-90.
33. Boehringer Ingelheim Pharmaceuticals Inc. Nevirapine Viramine Product Monography Ridgefield Connecticut. 2004.34.
35. Janssen-Cilag Ltd. Summary of Product Characteristics High Wycombe Bucks Intelence Janssen Cilag Ltd. 2008.
36. Sharma M, Saravolatz L. Rilpivirine: a new non-nucleoside reverse transcriptase inhibitor. Journal of Antimicrobial Chemotherapy. 2013;68 :250-6.
37. Full Prescribing Information Tibotec Edurant™ (Rilpivirine) Raritan, N J: Tibotec. Issued May, 2011.
38. Lamorde M, Walimbwa S, Byakika-Kibwika P, Katwere M, Mukisa L, Sempa JB, et al. Steady-state pharmacokinetics of Rilpivirine under different

meal conditions in HIV-1-infected Ugandan adults. Journal of Antimicrobial Chemotherapy. 2015;70(5):1482-6.

39. Molina JM, Cahn P, Grinsztejn B, Lazzarin A, Mills A, Saag M. Rilpivirine versus efavirenz with tenofovir and emtricitabine in treatment-naive adults infected with HIV-1 (ECHO): a phase 3 randomised double-blind active-controlled trial. Lancet. 2011;378: 238-46.

40. Wlodawer A, Erickson JW. Structure-based inhibitors of HIV‑1 protease. Annual Review of Biochemistry. 1993;62:543-85.

41. Hsu A, Granneman GR, Bertz RJ. Ritonavir. Clinical pharmacokinetics and interactions with other anti-HIV agents. Clinical Pharmacokinetics. 1998;35:275-91.

42. Croom KF, Dhillon S, Keam SJ. Atazanavir: a review of its use in the management of HIV-1 infection. Drugs. 2009;69: 1107-40.

43. M L, Byakika-Kibwika P, Boffito M, Nabukeera L, Mayito J, Ogwal-Okeng J, et al. Steady-state pharmacokinetics of lopinavir plus ritonavir when administered under different meal conditions in HIV-infected Ugandan adults. Journal of Acquired Immune Deficiency Syndromes. 2012;60(3):295-8.

44. Calza L. Renal toxicity associated with antiretroviral therapy. HIV Clinical Trials. 2012;13:189-211.

45. Brewster UC, Perazella MA. Acute interstitial nephritis associated with atazanavir, a new protease inhibitor. American Journal of Kidney Diseases. 2004;44 :81-4.

46. Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proceedings of the National Academy of Sciences. 2000;97 :5639-44.

47. Reeves JD, Coakley E, Petropoulos CJ, Whitcomb JM. An enhanced-sensitivity Trofile HIV co-receptor tropism assay for selecting patients for therapy with entry inhibitors targeting CCR5: A review of analytical and clinical studies. Journal of Viral Entry. 2009(3):94-102.

48. Lalezari J, Gathe J, Brinson C, Thompson M, Cohen C, Dejesus E, et al. Safety, efficacy, and pharmacokinetics of TBR-652, a CCR5/CCR2

antagonist, in HIV-1-infected, treatment-experienced, CCR5 antagonist-naive subjects. Journal of Acquired Immune Deficiency Syndromes. 2011;57 118-25.

49. Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, et al. Maraviroc (UK-427, 857), a potent, orally; bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum antihuman immunodeficiency virus type 1 activity. Antimicrobial Agents and Chemotherapy. 2005;49:4721-32.
50. Gulick R, Lalezari J, Goodrich JeaftMst. Maraviroc for previously treated patients with R5 HIV1 infection. New England Journal of Medicine. 2008;359 :1429-41.
51. Abel S, Back DJ, Vourvahis M. Maraviroc: pharmacokinetics and drug interactions. Antiviral Therapy. 2009;14(5):607-18.
52. Riddler SA, Haubrich R, DiRienzo AG. Class-sparing regimens for initial treatment of HIV-1 infection. New England Journal of Medicine. 2008;358 2095-106.
53. Gervasoni C, Cattaneo D. Pharmacokinetic concerns related to the Clinical Trial Group (ACTG) A5262 trial. AIDS. 2012;26 398-400.
54. Mora-Peris B, Croucher A, Else LJ, Vera JH, Khoo S, Scullard G, et al. Pharmacokinetic profile and safety of 150 mg of maraviroc dosed with 800/100 mg of Darunavir/ritonavir all once daily, with and without nucleoside analogues, in HIV-infected subjects. Journal of Antimicrobial Chemotherapy. 2013;68(6):1348-53.
55. Abel S, van Rosario MC, Ridgway CE, Medhurst CG, Taylor-Worth RJ, der Ryst E. Assessment of the pharmacokinetics, safety and tolerability of maraviroc, a novel CCR5 antagonist, in healthy volunteers. British Journal of Clinical Pharmacology. 2008;65(1):5-18.
56. Lennox JL, Dejesus E, Berger DS. Raltegravir versus efavirenz regimens in treatment-naive HIV-1-infected patients: 96-week efficacy, durability, subgroup, safety, and metabolic analyses. Journal of Acquired Immune Deficiency Syndromes. 2010;55 :39-48.
57. Sax PE, DeJesus E, Mills A. Co-formulated Elvitegravir, Cobicistat, emtricitabine, and tenofovir versus co-formulated efavirenz, emtricitabine, and tenofovir for initial treatment of HIV-1 infection: a randomised, double-

blind, phase 3 trial, analysis of results after 48 weeks. Lancet. 2012;379:2439-48.

58. Min S, Song I, Borland J. Pharmacokinetics and safety of S/GSK1349572, a next-generation HIV integrase inhibitor, in healthy volunteers. Antimicrobial Agents and Chemotherapy. 2010;54 :254-8.

59. Espeseth AS, Felock P, Wolfe A, Witmer M, Grobler J, Anthony N, et al. HIV-1 integrase inhibitors that compete with the target DNA substrate define a unique strand transfer conformation for integrase. Proceedings of the National Academy of Sciences. 2000;97:11244-9.

60. Grobler JA, Stillmock K, Hu B, Witmer M, Felock P, Espeseth AS, et al. Diketo acid inhibitor mechanism and HIV-1 integrase: Implications for metal binding in the active site of phosphotransferase enzymes. Proceedings of the National Academy of Sciences. 2002;99 :6661-6.

61. Brainard DM, Friedman EJ, Jin B. Effect of low-, moderate-, and high-fat meals on Raltegravir pharmacokinetics. Journal of Clinical Pharmacology. 2011;51(3):422-7.

62. Eron Jr JJ, Rockstroh JK, Reynes J. Raltegravir once daily or twice daily in previously untreated patients with HIV- a randomised, active-controlled, phase 3 non-inferiority trial. Lancet Infectious Diseases 2011;11:907-15.

63. Wenning LA, Petry AS, Kost JT. Pharmacokinetics of Raltegravir in individuals with UGT1A1 polymorphisms. Clinical Pharmacology Therapeutics. 2009;85(6):623-7.

64. Reese MJ, Savina PM, Generaux GT, Tracey H, Humphreys JE, Kanaoka E. In vitro investigations into the roles of drug transporters and metabolizing enzymes in the disposition and drug interactions of dolutegravir, a HIV integrase inhibitor. Drug Metabolism Disposition. 2013;41:353-61.

65. Lepist EI, Murray BP, Tong L, Roy A, Bannister R, Ray AS. Effect of cobicistat and ritonavir on proximal renal tubular cell uptake and efflux transporters. 51st Interscience Conference on Antimicrobial Agents and Chemotherapy ICAAC September Chicago Abstract A11724. 2011:17-20.

66. Gallant JE, Koenig E, Andrade-Villanueva J, Chetchotisakd P, DeJesus E, Antunes F, et al. Cobicistat versus ritonavir as a pharmacoenhancer of atazanavir plus emtricitabine/tenofovir disoproxil fumarate in treatment-naive

HIV type 1-infected patients: week 48 results. Journal of Infectious Diseases. 2013;208 :32-9.

67. German P, Liu HC, Szwarcberg J, Hepner M, Andrews J, Kearney BP, et al. Effect of cobicistat on glomerular filtration rate in subjects with normal and impaired renal function. Immune Defic Syndr. 2012;61:32-40.

68. Fellay J, Boubaker K. Prevalence of adverse events associated with potent antiretroviral treatment: Swiss HIV Cohort Study. Lancet. 2001;358: 1322-27.

69. Chowers MY, Gottesman BS, Leibovici L, Pielmeier U, Andreassen S, Paul M. Reporting of adverse events in randomized controlled trials of highly active antiretroviral therapy: systematic review. Journal of Antimicrobial Chemotherapy. 2009;64(2):239-50.

70. DAIDS (Division of AIDS, USA). Table for grading the severity of adult and pediatric adverse events, version 2. March Available from https://rsc.tech-res.com/docs/default-source/safety/daids-ae-grading-table-mar2017.pdf?sfvrsn=6

71. Naranjo CA, Busto U, Sellers EM. A method for estimating the probability of adverse drug reactions. Clinical Pharmacology Therapeutics. 1981;30:239-24.

72. Schulz KF, Altman DG, Moher D, Consort. CONSORT statement: updated guidelines for reporting parallel group randomized trials. Annals of Internal Medicine. 2010;152(11):726-32.

73. Ioannidis JP, Lau J. Completeness of safety reporting in randomized trials: an evaluation of 7 medical areas. Journal of American Medical Association. 2001;285: 437-43.

74. Carr A. Improvement of the study, analysis, and reporting of adverse events associated with antiretroviral therapy. Lancet. 2002;360: 81-5.

75. Adult AIDS Clinical Trial Group. Available at: http://aactg.s-3.

76. Nguyen ML, Nagy GS, Hernandez I, al e. Use of HAART in women: similar response but greater toxicity [abstract no WePeB5968]. 14th International AIDS conference July; Barcelona. 2002.

77. Pernerstorfer-Schoen H, Jilma B, Perschler A. Sex differences in HAART-associated dyslipidaemia. AIDS. 2001;15 725-34.

78. Soon GG, Min M, Struble KA, Chan-Tack KM, Birnkrant DB. Meta-analysis of gender differences in efficacy outcomes for HIV-positive subjects in

randomized controlled clinical trials of antiretroviral therapy (2000-2008). AIDS Patient Care STDS. 2012;26(8):2000-8.

79. Currier J, Spino C, Grimes J. Differences between women and men in adverse events and CD4+ responses to nucleoside analogue therapy for HIV infection. Journal of Acquired Immune Deficiency Syndromes. 2000;24:316-24.

80. Burger D, Van Der Heiden I LPCea. Gender-related differences in efavirenz pharmakokinetics [abstract no 15. Abstracts of the 4th International Workshop on Clinical Pharmacology of Mar Cannes. 2003:27-9.

81. Fletcher C. Gender differences in antiretroviral therapy pharmacokinetics and pharmacodynamics [online]. Available at: http://www.retroconference.org//pages/web for selected toxicities2004.

82. The Multicenter AIDS cohort study2002.

83. Anderson PL, Kakuda TN, Kawle S, Fletcher CV. Antiviral dynamics and sex differences of Zidovudine and lamivudine triphosphate concentrations in HIV infected individuals. AIDS. 2003;17 :2159-68.

84. Stretcher BN, Pesce AJ, Frame PT, Stein DS. Pharmacokinetics of Zidovudine phosphorylation in peripheral blood mononuclear cells from patients infected with human immunodeficiency virus. Antimicrobial Agents and Chemotherapy. 1994;38 :1541-7.

85. Moore RD, Fortgang I, Keruly J. Adverse events from drug therapy for Human Immuno Deficiency Virus Disease. American Journal of Medicine. 1996;101 :34-40.

86. Burger D VDHI, and La Porte C et al. Inter-patient variability in the pharmacokinetics of the HIV non-nucleoside reverse transcriptase inhibitor efavirenz: the effect of gender, race, and CYP2B6 polymorphism. British Journal of Clinical Pharmacology. 2006;61(2):148-54.

87. Warren KJ, Boxwell DE. Nevirapine-associated Stevens-Johnson syndrome. Lancet. 1998;351:567.

88. Spire B CP, Garzot MA, L'henaff M, Group T, Obadia Y. Factors associated with Efavirenz discontinuation in a large community-based sample of patients. AIDS Care. 2004;16 :558-64.

89. Kappelhoff BS, van Leth F, Robinson PA, MacGregor TR, Huitema AD, et al for the 2NN study group. Are adverse events of Nevirapine and efavirenz related to plasma concentrations. Antiviral Therapy. 2005;10(4):489-98.
90. La Porte C, Burger D, Gyssens I, Sprenger H, Koopmans P. Gender differences in Nevirapine pharmacokinetics, fact or fiction? Proceedings of the 4th International Workshop on Clinical Pharmacology of HIV therapy. Cannes France: Abstract 31. 2003.
91. Regazzi M, Villani P, Seminari E, Ravasi G, Cusato M, Marubbi F. Sex differences in Nevirapine disposition in HIV-infected patients. AIDS. 2003;17(16):2399-400.
92. Sanne I, Mommeja-Marin H, Hinkle J. Severe hepatotoxicity associated with nevirapine use in HI infected subjects. Journal of Infectious Diseases. 2005;191(6):825-9.
93. Hodder S, Arasteh K, Wet J, Gathe J, Gold J, Kumar P. Effect of gender and race on the week 48 findings in treatment-naive, HIV-1-infected patients enrolled in the randomized, phase III trials ECHO and THRIVE. HIV Medicine. 2012;13(7):406-15.
94. Burger DM MR, Van De Leur MR, La Porte CJL. Lopinavir plasma levels are significantly higher in female than in male HIV-1 infected patients. Proceedings of the 3rd International Workshop on Clinical Pharmacology of HIV therapy Washington DC, USA: Abstract 65. 2002.
95. Ofotokun I, Chuck SK, Binongo JN, Palau M, Lennox JL, Acosta EP. Lopinavir/Ritonavir pharmacokinetic profile: impact of sex and other covariates following a change from twice-daily to once-daily therapy. Journal of Clinical Pharmacology. 2007;47(8):970-7.
96. Fletcher CV, Jiang H, Brundage RC. Sex-based differences in saquinavir pharmacology and virologic response in Clinical Trials Group Study 359. Journal of Infectious Diseases. 2004;189(7):1176-84.
97. Burger DM, Siebers MC, Hugen PW, Aamoutse RE, Hekster YA, Koopmans PP. Pharmacokinetic variability caused by gender: do women have higher Indinavir exposure than men? Journal of Acquired Immune Deficiency Syndromes. 2002;29(1):101-2.

98. Arastéh K YP, Pozniak A, Grinsztejn Tomaka F.B et al. Efficacy and safety of darunavir/ritonavir in treatment-experienced HIV type-1 patients in the POWER 1, 2 and 3 trials at week 96. Antiviral Therapy. 2009;14(6):859-64.
99. Currier JS, Yetzer E, Pothoff A, Pasadena US. Gender differences in adverse events on ritonavir intolerance. Proceedings of National Conference on Women and HIV Abstract 3047. 1997.
100. Pai MP, Schriever CA, Diaz-Linares M, Novak RM, Rodvold KA. Sex-related differences in the pharmacokinetics of once-daily saquinavir soft-gelatin capsules boosted with low-dose ritonavir in patients infected with human immunodeficiency virus type 1. Pharmacotherapy. 2004;24:592-9.
101. Flexner CW, Chicago IL, Feinberg U. Factors associated with pharmacokinetic variability in HIV-infected patients. Northwestern of Medicine Office of Continuing Medical Education iMedOptions. 2004.
102. Rotger M, Glass TR, Junier T. Contribution of genetic background, traditional risk factors, and HIV-related factors to coronary artery disease events in HIV-positive persons. Clinical Infectious Diseases. 2013;57 :112-21.
103. Hoehe MR TB, Lehrach H. Human inter-individual DNA sequence variation in candidate genes, drug targets, the importance of haplotypes and pharmacogenomics. Current Pharmaceutical Biotechnology. 2003;4:351-78.
104. Abecasis GR, Auton A, Brooks LD. Genomes Project Consortium. An integrated map of genetic variation from 1092 human genomes. Nature. 2012;491: 56-65.
105. Biesecker LG, Green RC. Diagnostic clinical genome and exome sequencing. New England Journal of Medicine. 2014;370:2418-25.
106. Mallal S, Nolan D, Witt C. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor Abacavir. Lancet. 2002;359:727-32.
107. Rodriguez-Novoa S, Labarga P, Soriano V. Predictors of kidney tubular dysfunction in HIV positive patients treated with Tenofovir: a pharmacogenetic study. Clinical Infectious Diseases. 2009;48: 108-16.
108. Nishijima T, Komatsu H, Higasa K, Takano M, Gatanaga H. Single Nucleotide Polymorphisms in ABCC2 Associate With Tenofovir-Induced

Kidney Tubular Dysfunction in Japanese Patients With HIV-1 Infection: A Pharmacogenetic Study. Clinical Infectious Diseases. 2012;55(1).

109. Izzedine H, Hulot JS, Villard E, Goyenvalle C, Deray G. Association between ABCC2 Gene Haplotypes and Tenofovir-Induced Proximal Tubulopathy. Journal of Infectious Diseases. 2006;194 :1481-91.

110. Pushpakom SP, Liptrott NJ, Labarga P, Owen A. Rodríguez-Nóvoa S, and Pirmohamed M, et al. Genetic variants of ABCC10, a novel Tenofovir transporter, are associated with kidney tubular dysfunction. Journal of Infectious Diseases. 2011;204(1):145-53.

111. Kile DA, MaWhinney S, Aquilante CL, et al. A population pharmacokinetic, pharmacogenetic analysis of Atazanavir. AIDS Research and Human Retroviruses. 2012;28 :1227-34.

112. Johnson DH, Venuto C, Ritchie MD, et al. Genome wide association study of Atazanavir pharmacokinetics and hyperbilirubinemia in AIDS Clinical Trials Group protocol A5202. Pharmacogenetics and Genomics. 2014;24 :195-203.

113. Saag M, Balu R, Phillips E. High sensitivity of human leukocyte antigen-B*5701 as a marker for immunologically confirmed Abacavir hypersensitivity in white and black patients. Clinical Infectious Diseases. 2008;46 :1111-8.

114. Mallal S, Phillips E, Carosi G. HLA-B*5701 screening for hypersensitivity to Abacavir. New England Journal of Medicine. 2008;358 :568-79.

115. Nishijima T HT, Kurosawa T, Tanaka N, Oka S, Gatanaga H. Drug Transporter Genetic Variants Are Not Associated with TFV-Related Renal Dysfunction in Patients with HIV-1 Infection: A Pharmacogenetic Study. PLoS One. 2015;10(11).

116. Likanonsakul S SB, Nitiyanontakij R, Prasithsirikul W, Emi E. Nakayama, Tatsuo Shioda, Chariya Sangsajja. A Single-Nucleotide Polymorphism in ABCC4 Is Associated with Tenofovir-Related Beta2-Microglobulinuria in Thai Patients with HIV-1 Infection. PLoS One. 2016;11(1).

117. Hulgan T, Haas DW, Haines JL, Ritchie MD, Robbins GK, Shafer RW, et al. Mitochondrial haplogroups and peripheral neuropathy during antiretrovirals therapy: an adult AIDS clinical trials group study. AIDS. 2005;19: 1341-9.

118. Carr DF CM, Cornejo Castro EM, Jorgensen AL, Khoo S, Van Oosterhout JJ, Dandara C, Kampira E, Ssali F, Munderi P, Lalloo DG, Heyderman RS, and

Pirmohamed M. CYP2B6 c.983T>C polymorphism is associated with Nevirapine hypersensitivity in Malawian and Ugandan HIV populations. Journal of Antimicrobial Chemotherapy. 2014;69(12):3329-34.

119. Canter JA, Haas DW, Kallianpur AR, Ritchie MD, Hulgan T. The mitochondrial pharmacogenomics of haplogroup T: MTND2*LHON4917G and antiretroviral therapy-associated peripheral neuropathy. Pharmacogenomics Journal. 2008;8 71-7.
120. Holzinger ER, Grady B, Ritchie MD, et al. Genome-wide association study of plasma efavirenz pharmacokinetics in AIDS Clinical Trials Group protocols implicates several CYP2B6 variants. Pharmacogenetics and Genomics. 2012;22 :858-67.
121. Wyen C, Hendra H, Vogel H. Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. Journal of Antimicrobial Chemotherapy. 2008;61::914.
122. Saitoh A, Sarles E, Capparelli E. CYP2B6 genetic variants are associated with nevirapine pharmacokinetics and clinical response in HIV-1- infected children. AIDS. 2007;21: 2191-9.
123. Carr DF, Chaponda M, Jorgensen AL, et al. Association of human leukocyte antigen alleles and Nevirapine hypersensitivity in a infected population. Clinical Infectious Diseases. 2013;56: 1330-9.
124. Martin AM, Nolan D, James I. Predisposition to Nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. AIDS. 2005;19 :97-9.
125. Gatanaga H, Yazaki H, Tanuma J. HLA-Cw8 primarily associated with hypersensitivity to Nevirapine. AIDS. 2007;21 :264-5.
126. Ritchie MD, Haas DW, Motsinger AA, Donahue JP, Erdem H, Raffanti S, et al. Drug transporter and metabolizing enzyme gene variants and non-nucleoside reverse transcriptase inhibitor hepatotoxicity. Clinical Infectious Diseases. 2006;43 :779-82.
127. Haas DW, Bartlett JA, Andersen JW, Sanne I, Wilkinson GR, Hinkle J, et al. Pharmacogenetics of Nevirapineassociated hepatotoxicity An Adult Trials Group collaboration Clinical Infectious Diseases. 2006;43: 783-6.
128. Rodriguez-Novoa S BP, Rendón A, Barrios A, Corral A, Jiménez-Nacher I, González-Lahoz J, Soriano V. Plasma levels of Atazanavir and the risk of

hyperbilirubinemia are predicted by the 3435C/T polymorphism at the multi-drug resistance gene 1. Clinical Infectious Diseases. 2006;42:291-5.

129. Rotger M, Taffe P, Bleiber G, Gunthard HF, Furrer H, Vernazza P, et al. Swiss HIV Cohort Study. Gilbert syndrome and the development of antiretroviral therapyassociated hyperbilirubinemia Journal of Infectious Diseases. 2005;192: 1381-6.

130. Fauvel J BE, Ruidavets JB, Ferrières J, Toffoletti A, Massip P, Chap H, Perret B. An interaction between apo C-III variants and protease inhibitors contributes to high triglyceride/low HDL levels in treated HIV patients. AIDS. 2001;15 :2397-406.

131. Foulkes AS, Wohl DA, Frank I, Puleo E, Reilly MP. Association among race/ethnicity, APOC-III genotypes, and lipids in HIV-1-infected individuals on antiretrovirals therapy. PLos Medicine. 2006;3:337-47.

132. Tarr PE, Taffé P,, Bleiber G, Furrer H, Rotger M, Martinez R, Hirschel B, et al. Swiss HIV Cohort Study. Modeling the influence of APOC3 APOE and TNF polymorphisms on the risk of antiretroviral therapyassociated lipid disorders. Journal of Infectious Diseases. 2005;191: 1419-26.

133. Stohr W, Back D, Dunn D. Factors influencing efavirenz and Nevirapine plasma concentration: effect of ethnicity, weight and co-medication. Antiviral Therapy. 2008;13: 675-85.

134. Dickinson L, Chaponda M, Carr DF, van Oosterhout JJ, Kumwenda J, Lalloo DG, et al. Population pharmacokinetic and pharmacogenetic analysis of Nevirapine in hypersensitive and tolerant HIV-infected patients from Malawi. Antimicrobial Agents and Chemotherapy. 2014;58(2):706-12.

135. Shankarkumar U, Sridharan B, Pitchappan RM. HLA diversity among Nadars, a primitive Dravidian caste of South India. Tissue Antigens. 2003;62:542-7.

136. Pfister M, Labbe L, Hammer SM, Mellors J, Bennett KK, Rosenkranz S, et al. Population pharmacokinetics and pharmacodynamics of efavirenz, nelfinavir, and indinavir: Adult AIDS Clinical Trial Group Study 398. Antimicrobial Agents and Chemotherapy. 2003;47: 130-7.

137. Brundage RC, Yong FH, Fenton T, Spector SA, Starr SE, Fletcher CV. Intra-patient variability of efavirenz concentrations as a predictor of virologic

response to antiretroviral therapy. Antimicrobial Agents and Chemotherapy. 2004;48:979-84.

138. Stahle L, Moberg L, Svensson JO, Sonnerborg A. Efavirenz plasma concentrations in HIV-infected patients: inter-and intra-individual variability and clinical effects. Therapeutic Drug Monitoring. 2004;26:267-70.

139. Dhoro M, Zvada S, Ngara B, Nhachi C, Kadzirange G, Chonzi P, et al. CYP2B6*6, CYP2B6*18, Body weight, and sex are predictors of efavirenz pharmacokinetics and treatment response: population pharmacokinetic modeling in an HIV/AIDS and TB cohort in Zimbabwe. BMC Pharmacology and Toxicology. 2015;27 (16):4.

140. Chantarangsu E, Bartlett JA, Sanne I. Associations between HLA-DRB1*0102, HLA-B*5801, and hepatotoxicity during initiation of Nevirapine-containing regimens in South Africa. Journal of Acquired Immune Deficiency Syndromes. 2013;62: 55-7.

141. Ananworanich J, Moor Z. Incidence and risk factors for rash in Thai patients randomized to regimens with Nevirapine, Efavirenz or both drugs. AIDS. 2005;19(2):185-92.

142. Kearney BP, Yale K, Shah J, Zhong L, Flaherty JF. Pharmacokinetics and dosing recommendations of tenofovir disoproxil fumarate in hepatic or renal impairment. Clinical Pharmacokinetics. 2006;45(11):1115-24.

143. Ray AS, Cihlar T, Robinson KL. Mechanism of active renal tubular efflux of Tenofovir. Antimicrobial Agents and Chemotherapy. 2006;50:3297-304.

144. Gallant JE DE, Arribas JR, et al. Tenofovir DF, Emtricitabine, and Efavirenz vs Zidovudine, Lamivudine, and Efavirenz for HIV. New England Journal of Medicine. 2006;354:251-60.

145. Nelson M, Katlama C, Montaner J. The safety of Tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years. AIDS. 2007;21:1273-81.

146. Mocroft A, Kirk O, Reiss P. et al for the EUROSIDA group. Estimated glomerular filtration rate chronic kidney disease and antiretroviral drug use in HIVpositive patients AIDS. 2010;24 :1667-78.

147. Jones R, Stebbing J, Nelson M. Renal dysfunction with tenofovir disoproxil fumarate-containing highly active antiretroviral therapy regimens is not

observed more frequently: a cohort and case-control study. Journal of Acquired Immune Deficiency Syndromes. 2004;37:1489-95.

148. Franceschini N NS, Eron J Jr. Szczech LA, Finn WF. Incidence and aetiology of acute renal failure among ambulatory HIV infected patients. Kidney International. 2005;67:1526-31.

149. Padilla S, Gutierrez F, Masia M, Canovas V, Orozco C. Low frequency of renal function impairment during one-year of therapy with tenofovir-containing regimens in the real world: a case-control study. AIDS Patient Care, STDS. 2005;19 :421-4.

150. Miller D. Nucleoside phosphonate interactions with multiple organic anion transporters in renal proximal tubule. Journal of Pharmacology and Experimental Therapeutics. 2001;299: 567-74.

151. Imaoka T, Kusuhara H, Adachi M, Schuetz J, Takeuchi K. Functional involvement of multi-drug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. Molecular Pharmacology. 2007;71:619-27.

152. Moss DM, Neary M, Owen A. The role of drug transporters in the kidney: lessons from tenofovir. Frontiers in Pharmacology 2014;5 :248.

153. Kiser JJ, Carten ML, Aquilante CL, Anderson PL, Wolfe P, King TM. The effect of lopinavir/ritonavir on the renal clearance of tenofovir in HIV-infected patients. Clinical Pharmacology and Therapeutics. 2008;83 :265-72.

154. Cihlar T, Ray AS, Laflamme G, Vela JE, Tong L, Fuller MD. Molecular assessment of the potential for renal drug interactions between tenofovir and HIV protease inhibitors. Antiviral Therapy. 2007;12 :267-72.

155. Cihlar T, Laflamme G, Fisher R, Carey AC, Vela JE, Mackman R. Novel nucleotide human immunodeficiency virus reverse transcriptase inhibitor GS-9148 with a low nephrotoxic potential: characterization of renal transport and accumulation. Antimicrobial Agents and Chemotherapy. 2009;53 :150-6.

156. Lee WA, He GX, Eisenberg E, Cihlar T, Swaminathan S, Mulato A, Cundy KC. Selective intracellular activation of a novel prodrug of the human immunodeficiency virus reverse transcriptase inhibitor tenofovir leads to preferential distribution and accumulation in lymphatic tissue. Antimicrobial Agents Chemotherapy. 2005;49(5):1898-906.

157. Zolopa R, Sax P, Brar I, Elion R, Wang H, et al. Comparative study of Tenofovir alafenamide vs Tenofovir disoproxil fumarate each with Elvitegravir Cobicistat and Emtricitabine for HIV treatment. 20th Conference on Retroviruses and Opportunistic Infections Atlanta GA. 3-6 March, 2013.
158. Pozniak A, Arribas JR, Gathe J. Switching to Tenofovir Alafenamide, Coformulated With Elvitegravir, Cobicistat, and Emtricitabine, in HIV-Infected Patients With Renal Impairment: 48-Week Results From a Single-Arm, Multicenter, Open-Label Phase 3 Studyy. Journal of Acquired Immune Deficiency Syndromes. 2016;71(5):530-7.
159. Vaidya VS, Waikar SS, Ferguson MA. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clinical and Translational Science. 2008;1 :200-8.
160. Sabbisetti VS, Waikar SS, Antoine DJ, Smiles A, Bonventre JV. Blood kidney injury molecule-1 is a biomarker of acute and chronic kidney injury and predicts progression to ESRD in type I diabetes. Journal of the American Society of Nephrology. 2014;25(10):2177-86.
161. Hall AM, Edwards SG, Lapsley M, Connolly JO, Chetty K. Sub-clinical Tubular Injury in HIV-Infected Individuals on Antiretroviral Therapy: Analysis. . American Journal of Kidney Diseases. 2009;54 :1034-42.
162. Campbell LJ DT, Salota R, Cheserem E Post FA et al. Total protein, albumin and low-molecular-weight protein excretion in HIV-positive patients. BMC Nephrology. 2012;08(10):13-85.
163. Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV. Kidney injury molecule- a tissue and urinary biomarker for nephro-toxicant-induced renal injury. American Journal of Physiology Renal Physiology 286. 2004;1 :552-63.
164. Han WK, Bailly V, Abichandani R. Kidney injury molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. Kidney International. 2002;62 :237-44.
165. Shlipak MG, Scherzer R, Abraham A. Urinary Markers of Kidney Injury and Kidney Function Decline in HIV-Infected Women: Biomarkers and Kidney Decline. Journal of Acquired Immune Deficiency Syndromes. 2012;61 :565-73.

166. Nickolas TL, Schmidt-Ott KM, Canetta P. Diagnostic and prognostic stratification in the emergency department using urinary biomarkers of nephron damage: a multicenter prospective cohort study. Journal of American College of Cardiology. 2012;59 :246-55.
167. Peralta C SR, Grunfeld C, Abraham A, Shlipak M et al. Urinary biomarkers of kidney injury are associated with all-cause mortality in the Women's Interagency (WIHS). HIV Medicine 2014;15(5):291-300.
168. McLlroy DR, Wagner G, Lee HT. Biomarkers of acute kidney injury: an evolving domain. Anesthesiology. 2010;112 :998-1004.
169. Bernard AM, Lauwerys RR. Retinol binding protein in urine: a more practical index than urinary beta 2-microglobulin for the routine screening of renal tubular function. Clin Chemistry. 1981;27: 1781-2.
170. Calcagno A, Cusato J, Marinaro L, Simiele M, Bonora S. Tenofovir clearance is reduced in HIV-positive patients with subclinical tubular impairment. AIDS. 2015;30(6):915-20.
171. Ray AS, Wright MR, Rhodes GR. Lack of evidence for an effect of lopinavir/ritonavir on tenofovir renal clearance. Clinical Pharmacology and Therapeutics 2008;84(6):660.
172. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. The Journal of Biological Chemistry. 1993;268:10425-32.
173. Wagener G, Jan M, Kim M. Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. Anesthesiology. 2006;105: 485-91.
174. Paragas N, Nickolas TL, Wyatt C, Forster CS, Sise Mea. Urinary NGAL marks cystic disease in HIV-associated nephropathy. Journal of the American Society of Nephrology. 2009;20:1687-92.
175. Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A. Accuracy of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Diagnosis and Prognosis in Acute Kidney Injury: Review and Meta-analysis: American Journal of Kidney Diseases. 2009;54 :1012-24.
176. Bourbouze R, Baumann FC, Bonvalet JP, Farman N. Distribution of N-acetyl-beta-D-glucosaminidase isoenzymes along the rabbit nephron. Kidney International. 1984;25(4):636-42.

177. Tucker SM, Pierce RJ, Price RG. Characterisation of human N-acetyl-beta-D-glucosaminidase isoenzymes as an indicator of tissue damage in disease. Clinica Chimica Acta. 1980;102(1):29-40.
178. Scherzer R, Lin H, Abraham A, Thiessen-Philbrook H, Shlipak M. Use of urine biomarker-derived clusters to predict the risk of chronic kidney disease and all-cause mortality in HIV-infected women. Nephrology Dialysis Transplantation. 2016;31(9):1478-85.
179. Maatman RG, van Veerkamp JH, de Westerlo EM, van Kuppevelt TH. Molecular identification of the liver-and the heart-type fatty acid-binding proteins in human and rat kidney: Use of the reverse transcriptase polymerase chain reaction. Biochemical Journal. 1992;15 285-90.
180. Jabłonowska E WK, Piekarska A. Urine Liver-Type Fatty Acid-Binding Protein and Kidney Injury Molecule-1 in HIV-Infected Patients Receiving Combined Antiretroviral Treatment Based on Tenofovir. AIDS Research and Human Retroviruses. 2014;30(4):363-9.
181. Moher D, Liberati A, Tetzlaff J, Altman DG. The Preferred reporting items for systematic reviews and meta-analyses: The Prisma statement. . Annals of Internal Medicine. 2009;151(4):264-9.
182. Jorgensen AL, Williamson PR. Methodological quality of pharmacogenetic studies: issues of concern. Statistics in Medicine. 2008;27(30):6547-69.
183. Kiser JJ, Aquilante CL, Anderson PL, King TM, Carten ML, Fletcher CV. Clinical and genetic determinants of intracellular Tenofovir diphosphate concentrations in HIV-infected patients. Journal of Acquired Immune Deficiency Syndromes 2008;47(3):298-303.
184. Elens L, Tyteca D, Panin N, Courtoy P, Lison D, Demoulin JB, et al. Functional defect caused by the 4544G>A SNP in ABCC2: potential impact for drug cellular disposition. Pharmacogenetics and Genomics. 2011;21(12):884-93.
185. da Rocha IM GA, Lazzaretti RK, Notti RK, Sprinz E, and Mattevi VS. Polymorphisms associated with renal adverse effects of antiretroviral therapy in a Southern Brazilian HIV cohort. Pharmacogenetics and Genomics. 2015;25(11) :541-7.
186. Manosuthi W, Sukasem C, Thongyen S, Nilkamhang S, Sungkanuparph S. ABCC2*1C and plasma Tenofovir concentration are correlated to decreased

glomerular filtration rate in patients receiving a Tenofovir-containing antiretroviral regimen. Journal of Antimicrobial Chemotherapy. 2014;69 (8):2195-201.

187. Kiser JJ, Carten ML, Aquilante CL, Anderson PL, Wolfe P, King TM. The effect of Lopinavir/ritonavir on the renal clearance of Tenofovir in HIV positive patients. Clinical Pharmacology Therapeutics. 2008;83: 265-72.

188. Medicines and Healthcare Products Regulatory Agency/Commission on Human http://www.mhra.gov.uk.

189. Fux CA, Simcock M, Wolbers M, Bucher HC, Hirschel B, Opravil Mea. Tenofovir use is associated with a reduction in calculated glomerular filtration rates in the Swiss HIV cohort Study. Antiviral Therapy. 2007;12:1165-73.

190. Cao Y, Han Y, Xie J, Cui Q, Li T. Impact of a Tenofovir disoproxil fumarate plus ritonavir-boosted protease inhibitor-based regimen on renal function in HIV-infected individuals: a prospective, multicenter study. BMC Infectious Diseases. 2013;13(1):301.

191. Kearney BP, Mathias A, Mittan A, Sayre J, Ebrahimi R, Cheng AK. Pharmacokinetics and safety of tenofovir disoproxil Fumarate on co-administration with lopinavir/ritonavir. Journal of Acquired Immune Deficiency Syndromes. 2006;43: 278-83.

192. Wever K, van Agtmael MA, Carr A. Incomplete reversibility of tenofovir-related renal toxicity in HIV-infected men. Journal of Acquired Immune Deficiency Syndromes. 2010;55(1):78-81.

193. Uwai Y, Ida H, Tsuji Y, Katsura T, Inui K. Renal transport of Adefovir, Cidofovir, and Tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2). Pharmaceutical Research. 2007;24: 811-5.

194. Borst P, Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. Biochimica et Biophysica Acta. 1999;1461: 347-57.

195. Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. Cancer Letters. 2001;162(2):181-91.

196. Post FA, Moyle GJ, Stellbrink HJ, Domingo P, Lim ML. Randomized comparison of renal effects, efficacy, and safety with once-daily Abacavir/lamivudine versus tenofovir/emtricitabine, administered with

efavirenz, in antiretroviral-naive, HIV-1-infected adults: 48-week results from the ASSERT study. Journal of Acquired Immune Deficiency Syndromes. 2010;55(1):49-57.

197. Chaturvedi S, Farmer T, Kapke GF. Assay Validation for KIM-1: human urinary renal dysfunction biomarker. International Journal of Biological Sciences. 2009;5(2):128-34.

198. Xue WX, Yuanyuan; Wang, Qin; Xu, Weijia; Mou, Shan; Ni, Zhaohui. Diagnostic performance of urinary kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin for acute kidney injury in an obstructive nephropathy patient. Nephrology. 2014;19(4):186-94.

199. Bruggeman LA, Ross MD, Tanji N. Renal epithelium is a previously unrecognized site of HIV-1 infection. Journal of the American Society of Nephrology. 2000;11 :2079-87.

200. McWilliam SJ, Antoine DJ, Sabbisetti V, Pearce RE, Pirmohamed M. Biomarker Medicine. Reference intervals for urinary renal injury biomarkers KIM1 and NGAL in healthy children. 2014;8(10):1189-97.

201. Jotwani V, Scherzer R, Abraham A. Association of urine alpha1-microglobulin with kidney function decline and mortality in HIV-infected women. Clinical Journal of the American Society of Nephrology. 2015;10(1):63-73.

202. Arribas JR, Pozniak AL, Gallant JE, et al. Tenofovir disoproxil fumarate, emtricitabine, and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naive patients: 144-week analysis. Journal of Acquired Immune Deficiecy Syndrome. 2008;47:74-8.

203. Gallant JE, Winston JA, DeJesus E, et al. The 3-year renal safety of a tenofovir disoproxil fumarate vs. a thymidine analogue containing regimen in antiretroviral naive patients AIDS. 2008;22:2155-63.

204. Cassetti I, Madruga JV, Suleiman JM, et al. The safety and efficacy of tenofovir DF in combination with lamivudine and efavirenz through 6 years in antiretroviral-naive HIV-1-infected patients. HIV Clinical Trials. 2007;8: 164-72.

205. Cassetti I, Etzel A, Madruga JV, et al. The 10 year safety and efficacy of tenofovir disoproxil fumarate (TDF)-containing once-daily highly active

antiretroviral therapy (HAART). Journal of International AIDS Society. 2010;13:86.

APPENDICES

APPENDIX A: Study Collaborators

Principal investigator's	**Hospital/University site**
Dr. Frank Post	King's College Hospital London **Collaborated on TDF candidate SNP study (Chapter Four)**
Ms. Lucy Campbell	King's College Hospital London **Collaborated on TDF candidate SNP study (Chapter Four)**
Professor Joseph V Boventre	Renal Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA **Collaborated on the KIM-1 association study (Chapter Five)**

APPENDIX B: Case Record Form

Defining the prevalence and pattern of kidney injury in HIV-infected patients exposed to antiretroviral therapy (ART) drugs

Case Record Form: Cross sectional study/Prospective study

Date of assessment	Subject initials	Subject code	Date of Birth	Study number

Patient Information leaflet given to subject Yes ☐ No ☐

Consent Form signed Yes ☐ No ☐

Eligibility

	Yes	**No**
Age >18 years	☐	☐
Hiv-positive patients ”on” or ”off” Tenofovir	☐	☐
Any CD4 count or viral load	☐	☐
Able to give consent	☐	☐

Patient History……

Gender Male ☐ Female ☐

Ethnic group White ☐ Black ☐ Asian ☐ Other ☐

Past Medical History_____ Date __ /__ /__

Weight _____ kg Date __ / __ / __

Height _____ m Date __ / __ / __

Anti-retroviral Drug History

Please list current and all previous HIV drugs in regimens

Regimen	HIV drugs in Regimen (Dose not required)	Start date	Stop date	Date/time last ARV taken
1				
2				
3				
4				
5				
6				

Is patient currently taking any medication other than anti-retroviral therapy?

No ☐ Yes ☐

If YES please specify

Drug	Date started

Medical History **Current eGFR (date):**

Urine dipstick **Urine PCR (date):**

Urine ACR (date):

Past medical history: Hypertension/ Diabetes Mellitus / Smoker

Co-infection: Hepatitis B/ Hepatitis C

When CKD 1st diagnosed:

Relationship with ARVS: Definite Probable Possible Unlikely

If not related to ARVs, give likely cause:

APPENDIX C: DNA concentration

Extracted DNA concentration as estimated by spectrophotometry (NanoDrop®)

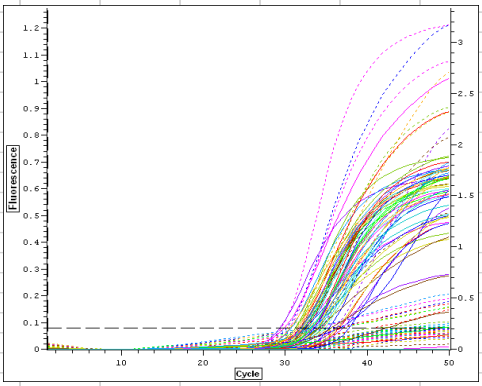
Sample ID	DNA Concentration (ng/µl)
GS18001	47.8
GS18002	8.6
GS18003	4.6
GS18004	6.2
GS18005	8.8
GS18006	4.8
GS18007	7.9
GS18008	9.1
GS18009	7.4
GS18010	4.6
GS18011	6.6
GS18012	8
GS18013	3.9
GS18014	3.2
GS18015	4.8
GS18016	3.9
GS18017	2.1
GS18018	4
GS18019	5.7
GS18020	10.9
GS18021	7.6
GS18022	6.5
GS18023	4.7
GS18024	18.1
GS18025	9.8
GS18026	6.9
GS18027	13.1
GS18028	13.1
GS18029	12.1
GS18030	16.3
GS18031	8
GS18032	7.7
GS18033	4.2
GS18034	5.4
GS18035	5.2
GS18036	4.5
GS18037	5.1
GS18038	4.2
GS18039	6.1
GS18040	6.5
GS18041	7.4
GS18042	5.6
GS18043	2.7

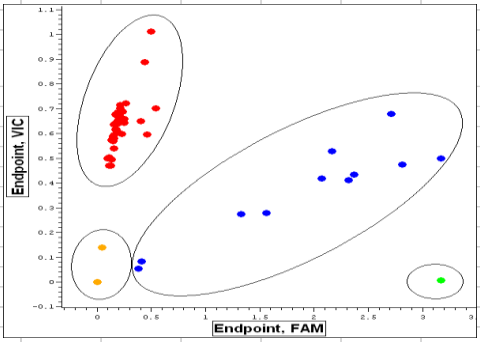
GS18044	5.4
GS18045	5
GS18046	8.6
GS18047	4.6
GS18048	10.1
GS18049	6.8
GS18050	7.3
GS18051	4
GS18052	4.4
GS18053	5.9
GS18054	4.6
GS18055	5.9
GS18056	6.6
GS18057	5.6
GS18058	6
GS18059	7.6
GS18060	7.3
GS18061	4
GS18062	6.5
GS18063	3.8
GS18064	7.2
GS18065	5.6
GS18066	8.3
GS18067	7.7
GS18068	4.8
GS18069	6
GS18070	6.2
GS18071	11.4
GS18072	1.2
GS18073	5.9
GS18074	5.5
GS18075	12.1
GS18076	7.4
GS18077	66.4
GS18078	3.1
GS18079	6.9
GS18080	7.2
GS18081	4.1
GS18082	1.8
GS18083	6.5
GS18084	18.2
GS18085	1.3
GS18086	18
GS18087	1.2
GS18088	1.7
GS18089	19.9
GS18090	0.8
GS18003	47.8

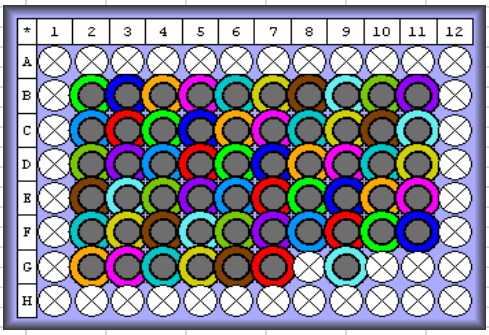
GS18004	8.6
GS18005	4.6
GS18006	6.2
GS18007	8.8
GS18008	4.8
GS18009	7.9
GS18010	9.1
GS18011	7.4
GS18012	4.6

Appendix D

PCR raw data including amplifications plots and reaction plate samples



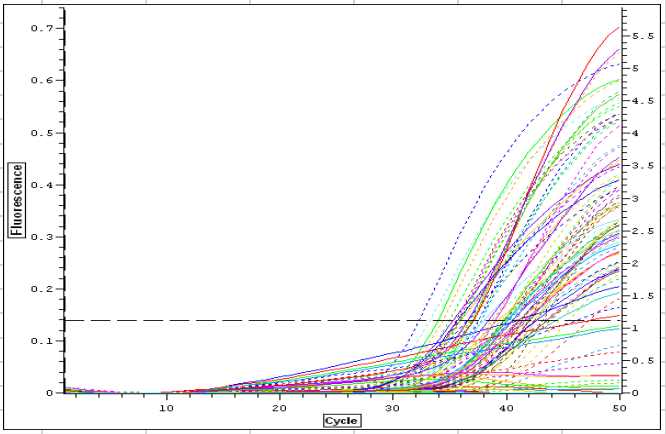


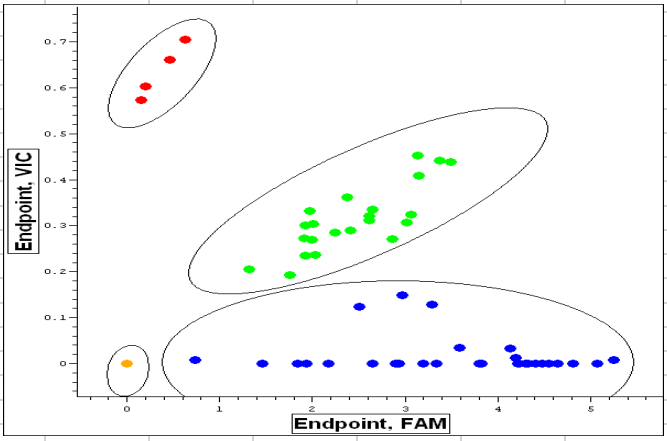


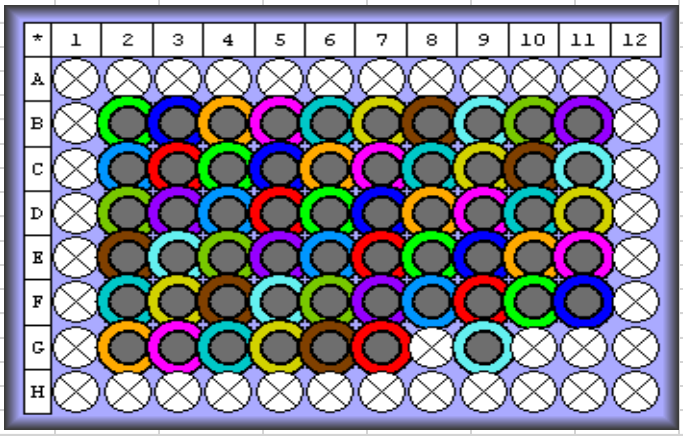
ABCC2_24_CC_GS18057_GS18112

Well	Group	X Content	Y Content
B2	Mutant		
B3	Mutant		
B4	Mutant		
B5	Heterozygous		
B6	Mutant		
B7	Mutant		
B8	Mutant		
B9	Mutant		
B10	Heterozygous		
B11	Mutant		
C2	Mutant		
C3	Mutant		
C4	Mutant		
C5	Heterozygous		
C6	Mutant		
C7	Mutant		
C8	Mutant		
C9	Mutant		
C10	Mutant		
C11	Mutant		
D2	Mutant		
D3	Heterozygous		
D4	Mutant		
D5	Mutant		
D6	Heterozygous		
D7	Mutant		
D8	Heterozygous		
D9	Mutant		
D10	Mutant		
D11	Heterozygous		
E2	Blank		
E3	Mutant		
E4	Mutant		
E5	Heterozygous		
E6	Mutant		
E7	Mutant		
E8	Mutant		
E9	Mutant		
E10	Mutant		
E11	Wild		
F2	Mutant		
F3	Mutant		
F4	Heterozygous		
F5	Mutant		
F6	Mutant		
F7	Mutant		
F8	Mutant		
F9	Mutant		
F10	Mutant		
F11	Mutant		
G2	Mutant		
G3	Mutant		
G4	Mutant		
G5	Mutant		
G6	Heterozygous		
G7	Heterozygous		
G9	Blank		

ABCC4_A3463G (rs1751034)

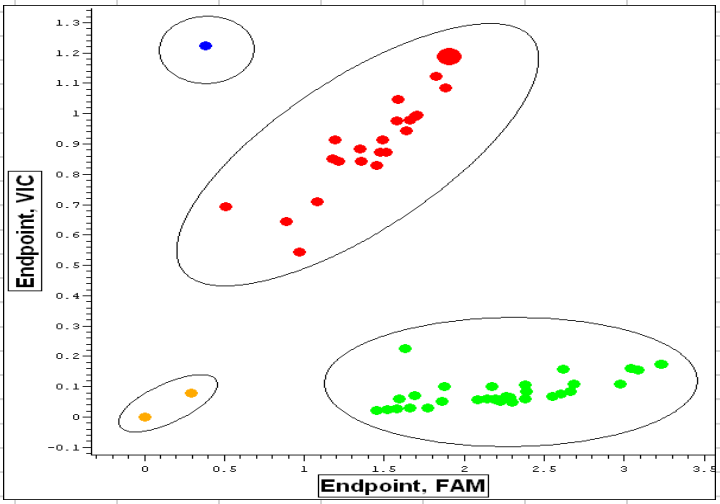


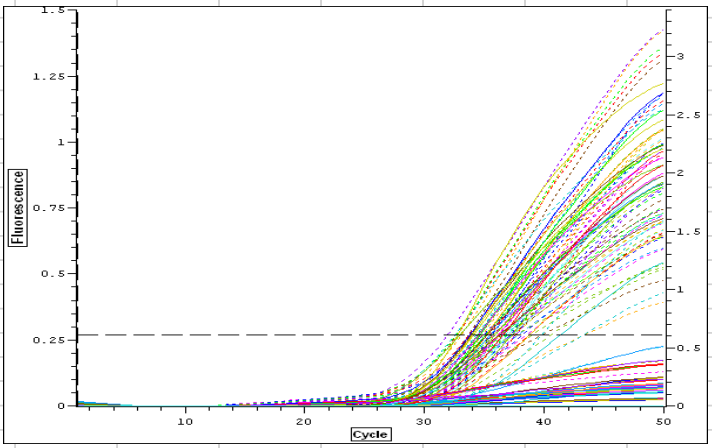


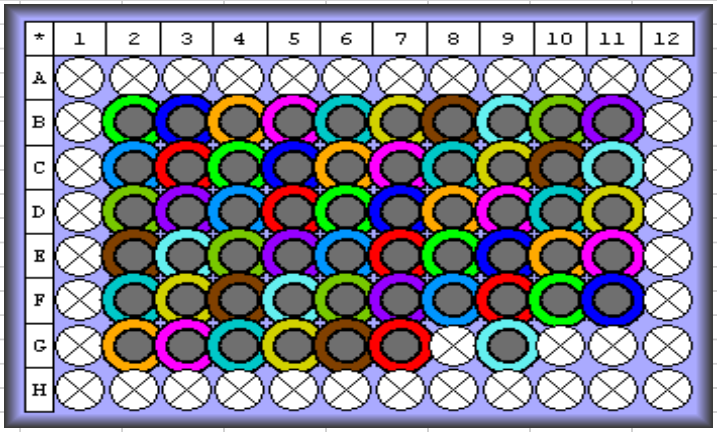


Well	Group	X Content	Y Content
B2	Wild		
B3	Wild		
B4	Heterozygous		
B5	Wild		
B6	Heterozygous		
B7	Wild		
B8	Wild		
B9	Heterozygous		
B10	Mutant		
B11	Heterozygous		
C2	Wild		
C3	Wild		
C4	Mutant		
C5	Wild		
C6	Wild		
C7	Wild		
C8	Wild		
C9	Heterozygous		
C10	Wild		
C11	Wild		
D2	Wild		
D3	Heterozygous		
D4	Wild		
D5	Mutant		
D6	Wild		
D7	Heterozygous		
D8	Wild		
D9	Heterozygous		
D10	Heterozygous		
D11	Heterozygous		
E2	Heterozygous		
E3	Heterozygous		
E4	Heterozygous		
E5	Heterozygous		
E6	Wild		
E7	Wild		
E8	Wild		
E9	Heterozygous		
E10	Heterozygous		
E11	Heterozygous		
F2	Wild		
F3	Heterozygous		
F4	Wild		
F5	Wild		
F6	Wild		
F7	Mutant		
F8	Heterozygous		
F9	Wild		
F10	Wild		
F11	Heterozygous		
G2	Wild		
G3	Wild		
G4	Heterozygous		
G5	Wild		
G6	Heterozygous		
G7	Wild		
G9	Blank		

ABCC4_669_C_T (rs899494)

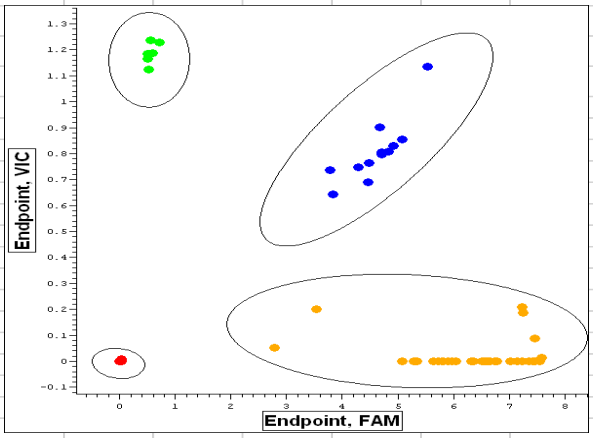


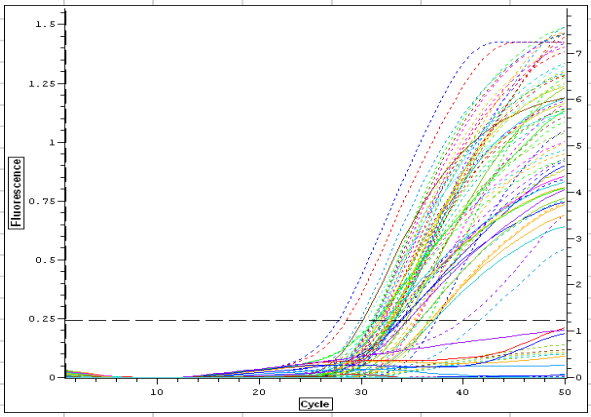


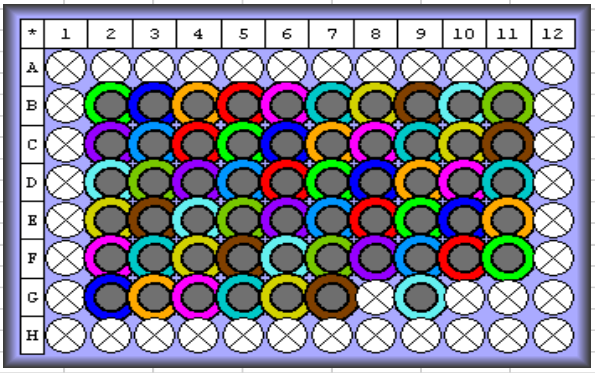


Well	Group	X Content
B2	Heterozygous	
B3	Wild	
B4	Heterozygous	
B5	Wild	
B6	Wild	
B7	Wild	
B8	Wild	
B9	Wild	
B10	Heterozygous	
B11	Wild	
C2	Wild	
C3	Wild	
C4	Heterozygous	
C5	Wild	
C6	Heterozygous	
C7	Blank	
C8	Wild	
C9	Wild	
C10	Heterozygous	
C11	Heterozygous	
D2	Wild	
D3	Wild	
D4	Heterozygous	
D5	Wild	
D6	Wild	
D7	Heterozygous	
D8	Wild	
D9	Heterozygous	
D10	Heterozygous	
D11	Heterozygous	
E2	Wild	
E3	Wild	
E4	Heterozygous	
E5	Wild	
E6	Wild	
E7	Wild	
E8	Heterozygous	
E9	Wild	
E10	Wild	
E11	Wild	
F2	Wild	
F3	Mutant	
F4	Heterozygous	
F5	Heterozygous	
F6	Heterozygous	
F7	Wild	
F8	Wild	
F9	Heterozygous	
F10	Heterozygous	
F11	Wild	
G2	Wild	
G3	Heterozygous	
G4	Wild	
G5	Heterozygous	
G6	Heterozygous	
G7	Wild	
G9	Blank	

ABCC10_(rs9349256) _GS18001_GS18056



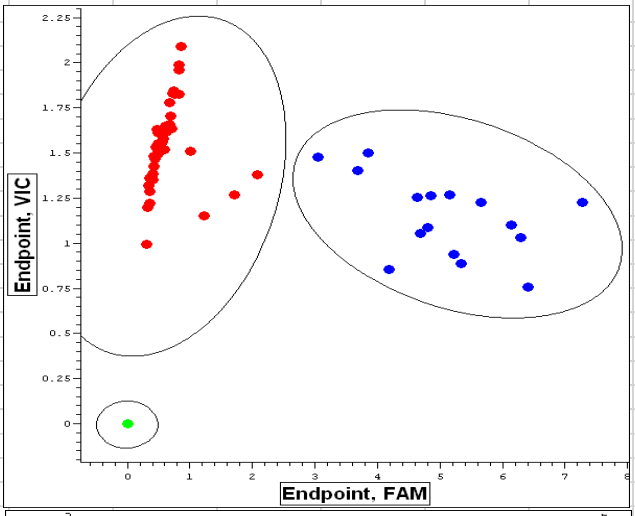


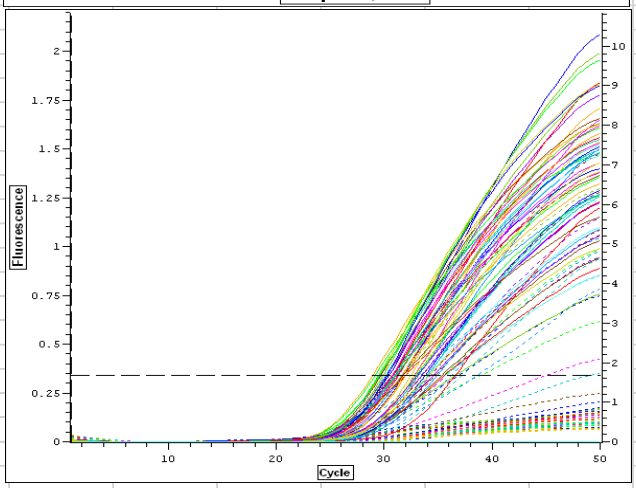


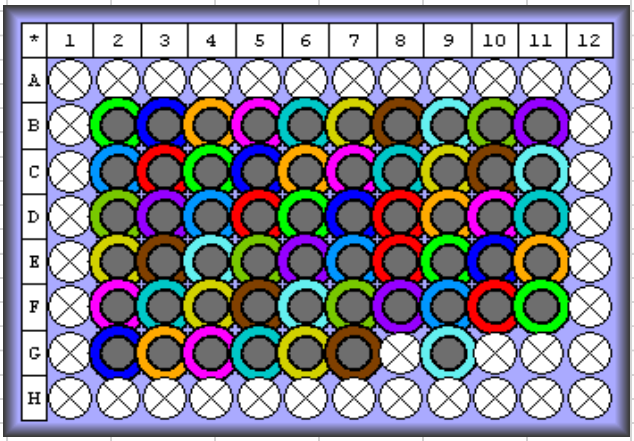
Ancestral Allele = GG FAM = G
Mutant = AA VIC = A
Heterozygous = AG

Well	Group	X Content	Y Content
B2	Mutant		
B3	Heterozygous		
B4	Heterozygous		
B5	Mutant		
B6	Blank		
B7	Mutant		
B8	Wild		
B9	Mutant		
B10	Mutant		
B11	Mutant		
C2	Mutant		
C3	Mutant		
C4	Mutant		
C5	Mutant		
C6	Heterozygous		
C7	Mutant		
C8	Mutant		
C9	Wild		
C10	Mutant		
C11	Wild		
D2	Wild		
D3	Heterozygous		
D4	Mutant		
D5	Mutant		
D6	Mutant		
D7	Heterozygous		
D8	Mutant		
D9	Heterozygous		
D10	Heterozygous		
D11	Heterozygous		
E2	Mutant		
E3	Mutant		
E4	Mutant		
E5	Wild		
E6	Mutant		
E7	Mutant		
E8	Mutant		
E9	Mutant		
E10	Blank		
E11	Mutant		
F2	Mutant		
F3	Mutant		
F4	Wild		
F5	Mutant		
F6	Mutant		
F7	Mutant		
F8	Heterozygous		
F9	Mutant		
F10	Mutant		
F11	Heterozygous		
G2	Mutant		
G3	Heterozygous		
G4	Mutant		
G5	Heterozygous		
G6	Mutant		
G7	Mutant		
G9	Blank		

SLC22A6 453A (G>A)







Well	Group	X Content	Y Content
B2	Heterozygous		
B3	Heterozygous		
B4	Wild		
B5	Wild		
B6	Wild		
B7	Wild		
B8	Wild		
B9	Heterozygous		
B10	Heterozygous		
B11	Wild		
C2	Wild		
C3	Heterozygous		
C4	Heterozygous		
C5	Wild		
C6	Wild		
C7	Wild		
C8	Wild		
C9	Wild		
C10	Heterozygous		
C11	Wild		
D2	Wild		
D3	Heterozygous		
D4	Heterozygous		
D5	Wild		
D6	Wild		
D7	Wild		
D8	Heterozygous		
D9	Wild		
D10	Wild		
D11	Wild		
E2	Wild		
E3	Wild		
E4	Wild		
E5	Wild		
E6	Wild		
E7	Heterozygous		
E8	Wild		
E9	Wild		
E10	Wild		
E11	Wild		
F2	Wild		
F3	Wild		
F4	Wild		
F5	Heterozygous		
F6	Heterozygous		
F7	Wild		
F8	Heterozygous		
F9	Heterozygous		
F10	Wild		
F11	Wild		
G2	Wild		
G3	Wild		
G4	Wild		
G5	Heterozygous		
G6	Wild		
G7	Wild		
G9	Blank		